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THE VITAMINE CONTENT OF BREWERS' YEAST.

By ATHERTON SEIDELL.

(From the Hygienic Laboratory, United States Public Health Service, Washington, D. C.)

(Received for publication, January 12, 1917.)

On account of the relation between the consumption of polished rice and the disease beri-beri, the early conceptions of vitamine were for the most part obtained from studies of rice polishings. It was soon shown, however, that curative fractions could be obtained from other sources. Among these ordinary brewers' yeast was found to be relatively rich in vitamine, and since it is a product which has so far not been extensively utilized in other ways, it may be considered a favorable raw material for vitamine studies.

Bottom yeast remaining after fermentation of the beer consists of a thick cream-colored mush. On warming, it rapidly expands with the evolution of carbon dioxide. The residual beer in it can be readily removed by pressing in a hydraulic or other suitable press. The amount of liquid thus removed is equal to about one-third to one-half of the weight of the original mass. The press cake when dried in a rapid current of air yields brownish brittle lumps which, if kept in a dry place, apparently remain unchanged for long periods. If the fresh press cake is warmed to a temperature of about 37.5° C. for 24 to 48 hours, it autolyzes more or less completely and yields a thick slimy liquid. This latter can be filtered only with great difficulty. A clear filtrate can best be obtained from it on a laboratory scale by use of large glass funnels and folded filter papers. It has been found that the yield of clear red-brown filtrate corresponds usually to about one-half the weight of the thick unfiltered liquid. The residue on the filter no doubt retains a considerable amount of the vitamine, but so far a satisfactory method for making a more complete separation of the liquid and undigested solids has not been found. If autolyzed yeast filtrate is kept in a cool place, it apparently remains unaltered for several months, except for the gradual deposition of a sediment, which appears to be greater the lower the temperature.

Activity of Autolyzed Yeast Filtrate.—In order to ascertain what quantity of autolyzed yeast filtrate is required to counter-

balance the deficiency of an exclusive diet of polished rice, the following feeding experiments were made on pigeons. The results are shown in Charts 1 and 3.[1] For the sake of comparison, the data for a large number of control pigeons fed on polished rice at various times during the course of the investigation are given in Chart 2.

Briefly, the experiments were made in all cases as follows: Groups of pigeons were kept in cages and given only water and polished rice. Each pigeon was weighed every 2nd or 3rd day, and the designated amount of the yeast filtrate administered with the aid of a small rubber tube. The figures for the abscissæ in the charts show the percentage change in weight as found by dividing the observed weight from day to day by the average weight of the bird for several days just preceding the experiment. The ordinates show the days the experiments were continued. From these charts it is seen that whereas control birds (Chart 2) receiving only polished rice decline to about 65 to 75 per cent of their original weight and develop typical polyneuritis within about 15 to 30 days, the birds which received doses of as little as 1.0 cc. of yeast filtrate (Chart 1) on alternate days lived for at least 3 months with, in two cases, no significant change in weight, and in a third a loss of approximately 25 per cent of its original weight, without showing symptoms of polyneuritis. The birds receiving 3 cc. doses of the yeast filtrate, except in one case, which was probably a young growing bird, gained practically no more in weight than the birds receiving 1 cc. doses of the filtrate. It is probable, therefore, that more than the minimum amount of vitamine required to maintain normal weight is not utilized in the organism.

In the case of the bird which received 0.5 cc. of the autolyzed yeast filtrate on alternate days (Chart 1), a marked loss in weight occurred. Unfortunately this bird died on the 43rd day, probably of some other cause than polyneuritis. The bird which received 0.3 cc. of the filtrate on alternate days proceeded in the typical manner to the development of polyneuritis on the 38th day.

[1] The data for Chart 3 were kindly furnished by Mr. R. R. Williams, of the Bureau of Chemistry.

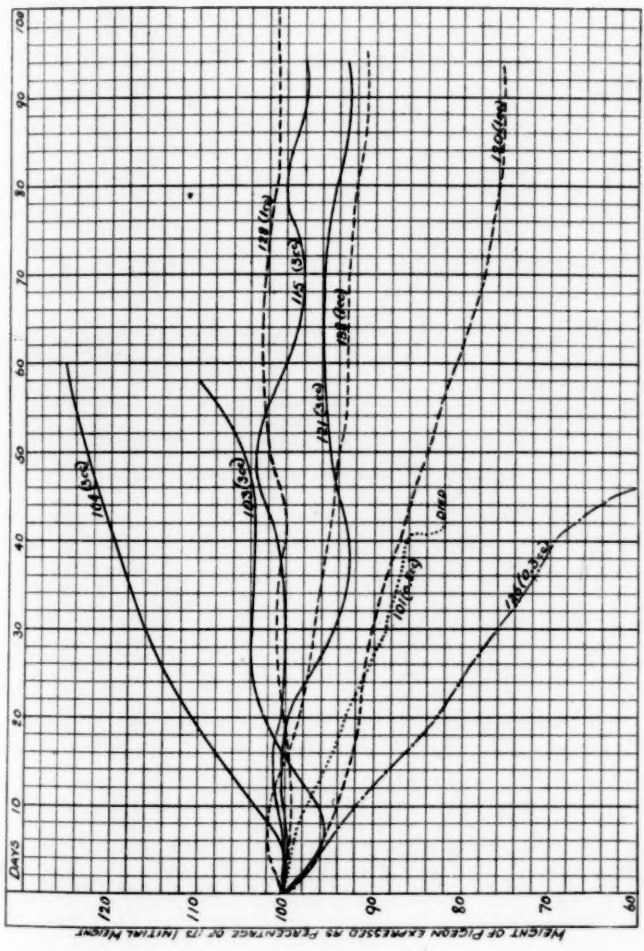

CHART 1. The change in weight of pigeons fed on polished rice and given the indicated quantities of autolyzed yeast filtrate on alternate days. The termination of the line, except for Pigeons 101 and 126 which died as shown, indicates the discontinuation of the experiment. The actual weighings give more or less irregular points (as shown in Chart 3) due to the large quantities of food which pigeons may take into their crops at irregular intervals.

Considering the results shown in Chart 3 it is seen that 1.0 cc. doses of the sample of yeast filtrate here used failed to protect the birds against a rapid loss in weight. This sample had been

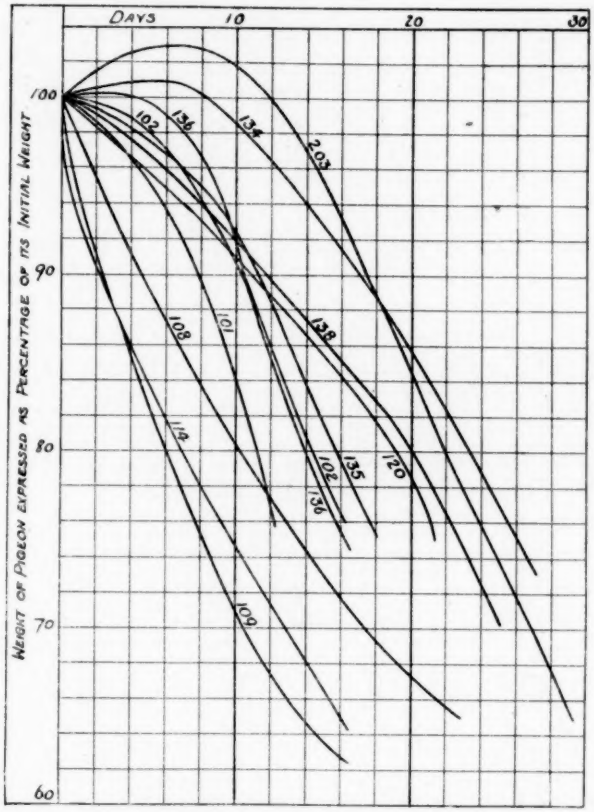

CHART 2. The rates of decline in weight of pigeons fed exclusively on polished rice. The termination of the lines indicates development of typical polyneuritic paralysis or death of the pigeon. The curves represent the average loss in weight. The actual points show irregularities due to daily variation in quantity of food eaten by the pigeon.

kept in a cool place for several months preceding its use and may have suffered some deterioration or may possibly have contained less vitamine in the first place than was present in the filtrate used in the experiment recorded in Chart 1. It is of

interest to note that an increase of the dose to 2.0 cc. soon overcame the deficiency and caused an increase in the weight of the birds. This experiment appears to indicate that a comparatively slight deficiency from the minimum daily vitamine requirements will result in a perceptible loss in weight, and the restoration of this deficiency will be eventually followed by an increase in the weight.

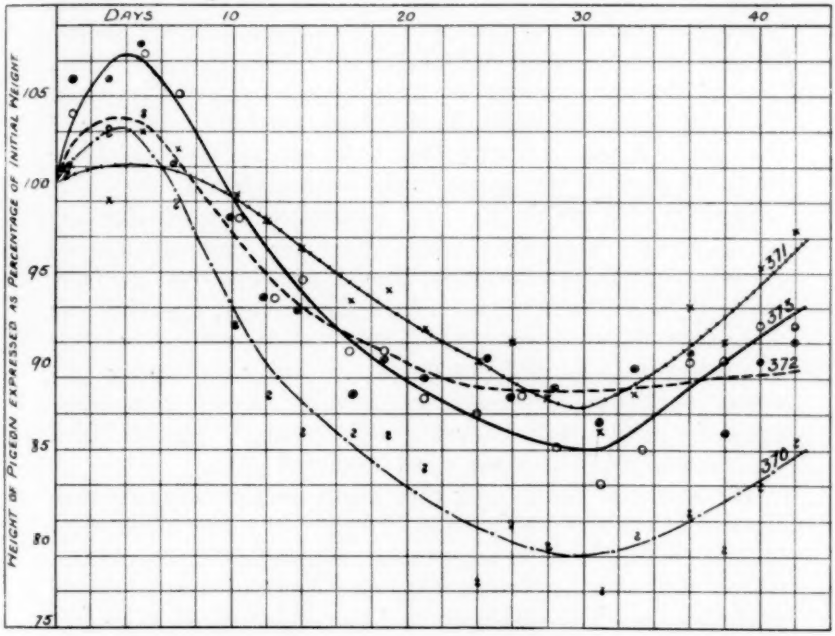

CHART 3. The change in weight of pigeons fed on polished rice and given doses of autolyzed yeast filtrate on alternate days. Each bird received 1.0 cc. doses from the 1st to the 17th day and 2.0 cc. doses thereafter. The actual weighings are indicated on the chart. The lines are intended to represent the average of these points.

From these results it is apparent that less than 1.0 cc. of autolyzed yeast filtrate given on alternate days to a pigeon fed only on polished rice is insufficient to prevent loss in weight and eventual development of polyneuritis. This quantity may in some cases be less than enough to prevent loss in weight, and in others be entirely adequate. It is, therefore, probable that the quan-

tity of vitamine in 0.5 cc. of filtrate from autolyzed pressed brewers' yeast, freshly prepared as described above, is just sufficient to supply the daily vitamine requirement of a grown pigeon of average size.

Activity of Dried Yeast.—In order to compare the activity of dried yeast with that of the autolyzed material, a feeding experiment similar to the above was made with several samples of dried yeast. Of these, one was prepared in the laboratory by simply blowing a rapid current of air over a thin layer of freshly pressed brewers' yeast for about 24 hours or until it was dry and brittle. Two of the samples were kindly prepared by the Wittemann Company of Buffalo, with the aid of an apparatus consisting of two steam heated rotating drums (steam pressure averaging about 35 pounds per square inch) between which the yeast passes and from which the dried material is scraped after being in contact with the heated drums for approximately 40 seconds. Of these two samples, one was prepared from the fresh brewers' yeast just as obtained from the brewery, the other was made from brewers' yeast which was first allowed to autolyze in a warm place for 48 hours previous to being put through the drying apparatus. The samples described above were designated, for convenience, laboratory dried yeast, Wittemann dried fresh yeast, and Wittemann dried autolyzed yeast.

The results of the feeding experiment on pigeons made with these three samples are shown in Chart 4. The dose of the dried products selected for this experiment was estimated to be approximately equivalent to the quantity, *viz.*, 1.0 cc. of clear autolyzed yeast filtrate, which, as shown in Chart 1, appeared to be just sufficient to replace the deficiency of a polished rice diet. Total solids determinations on autolyzed yeast filtrate (sp. gr. 1.06 at 15°C.) showed an average of about 0.2 gm. per 1.0 cc. It would be expected that the unfiltered autolyzed yeast (which of course is equivalent weight for weight to fresh pressed yeast) would contain approximately 0.25 gm. of dry residue per 1.0 cc. Direct determination on freshly pressed yeast showed a moisture content of about 75 per cent. It appears, therefore, reasonable to assume that the 0.25 gm. dose of dried yeast used in the experiment is approximately equivalent in potential vitamine to 1.0 cc. of autolyzed yeast filtrate.

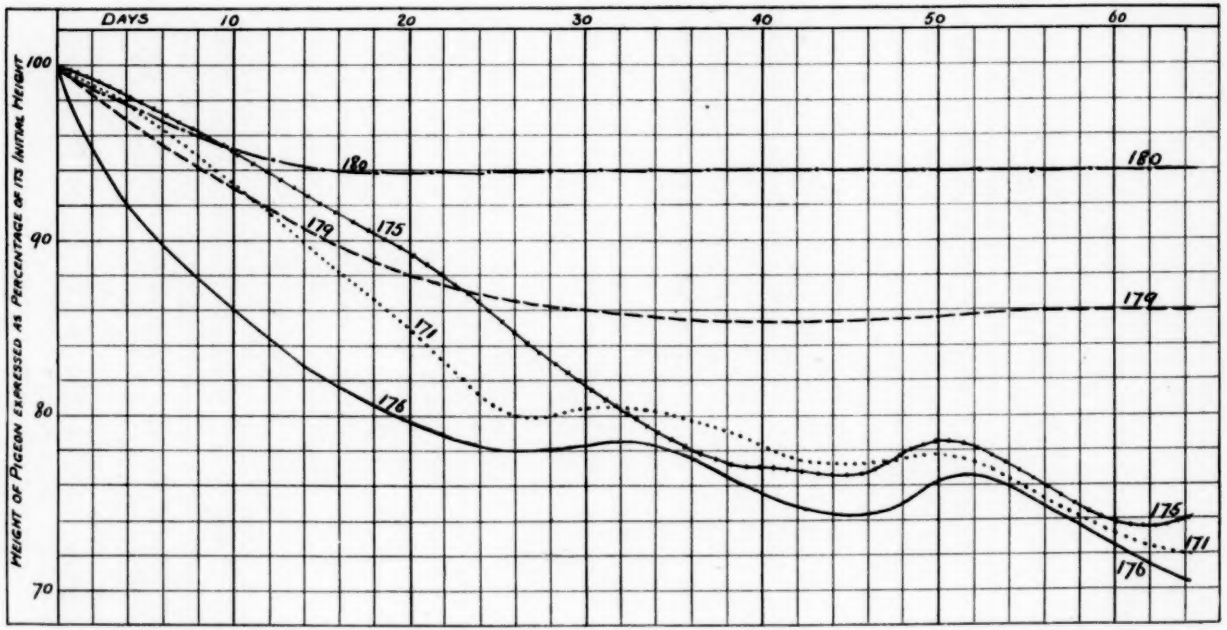

CHART 4. The change in weight of pigeons fed on polished rice and given 0.25 gm. doses of dried yeast on alternate days. Bird 171 received the laboratory dried product. Birds 175 and 176 received the Wittemann dried fresh yeast, and Birds 179 and 180 received the Wittemann dried autolyzed yeast.

On comparing the weight curves in Chart 4 with those in Chart 1, it is seen that considerably less protection was afforded by the dried products than was found for presumably equivalent doses of the clear liquid filtrate. It seems, therefore, probable that to replace a given diet deficiency somewhat larger quantities of dried yeast than of autolyzed yeast are required.

Influence of Autolysis upon Yeast Vitamine.—Considering further the results shown in Chart 4, it is seen that Birds 171, 175, and 176, which received the dried fresh yeast, lost considerably more weight on a rice diet than Birds 179 and 180, which received the dried autolyzed product. The only difference in the two cases, as already pointed out, was that one lot of the brewers' yeast was allowed to autolyze before being dried and the other was not. The experiment appears, therefore, to show conclusively that autolysis increases the protection afforded by the vitamine of brewers' yeast.

Additional evidence confirming this conclusion was obtained from another feeding experiment in which doses of 0.05 gm. of the dried products were used. This amount was far too small to afford more than a slight protection, but it is interesting to note that the two birds receiving the autolyzed product lost weight distinctly less rapidly than the two receiving the freshly dried material.

Estimation of the Quantity of Vitamine Isolated from Yeast Filtrate by Adsorption to Fullers' Earth.[2]—Since it has been shown by previous experiments[3] that fullers' earth selectively adsorbs vitamine from autolyzed yeast filtrate, it was thought that advantage of this property could be taken to separate the vitamine from the greater part of the other nitrogenous compounds which accompany it and obtain an approximate estimate of its quantity by determination of the nitrogen retained by the fullers' earth.

Samples of fullers' earth activated by being shaken with autolyzed yeast filtrate were found to contain approximately 2 per cent of nitrogen. The question arises, however, as to whether this nitrogen represents only vitamine or a mixture of vitamine and other nitrogenous compounds.

[2] The particular variety of fullers' earth so far used in this work has been generously supplied by Professor John U. Lloyd of Cincinnati, and is characterized by its exceptionally high adsorptive capacity for alkaloids.

[3] Seidell, A., *U. S. Pub. Health Reports*, 1916, xxxi, 364.

To obtain data on this point, 100 cc. of a sample of autolyzed yeast filtrate prepared as previously described, were shaken for several minutes with 5.0 gm. of fullers' earth, and the solid was removed by filtration. The clear liquid was then shaken with a second 5.0 gm. portion of the fullers' earth, the mixture filtered, and this procedure repeated a third time. Nitrogen determinations made on the original yeast filtrate and on each of the samples of fullers' earth which had been shaken in succession with the liquid, gave the following results. The original liquid contained 2.0 gm. of nitrogen per 100 cc. The first 5.0 gm. portion of fullers' earth contained 0.090 gm. of nitrogen, the second 0.073 gm., and the third 0.058 gm.

Experiments reported in the paper referred to above showed that with a ratio of 5 gm. of solid per 100 cc. of liquid, practically all the vitamine is removed from yeast filtrate. From the present results, which show that additional nitrogen is adsorbed by further treatment with fullers' earth, it is apparent that vitamine is only one of perhaps several nitrogenous compounds removable from yeast filtrate by fullers' earth. Although according to the previous experiments all the vitamine appears to be in the first fraction, it does not follow that other nitrogenous compounds are absent from this fraction. In fact it would be expected that some are adsorbed simultaneously with the vitamine. Therefore, the 0.09 gm. of nitrogen in the first lot of fullers' earth is probably representative of both vitamine and other nitrogenous compounds. How great a percentage of the latter is present can, of course, not be judged until further experiments are made. In the meantime it has been thought of interest to neglect the presence of any other nitrogenous compounds and assume that the nitrogen found represents only vitamine. In this way an approximate estimate of the maximum quantity of vitamine in yeast filtrate will be obtained.

From recent experiments by Williams and Seidell[4] it seems probable that vitamine contains approximately 50 per cent of nitrogen. On this basis, if it is assumed that all the 0.09 gm. of nitrogen in the first 5.0 gm. of fullers' earth shaken with 100 cc. of yeast filtrate as described above is vitamine nitrogen, it follows that 0.18 gm. per 100 cc. of autolyzed yeast filtrate is the maximum amount of vitamine which can be present.

[4] Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 431.

The feeding experiments on pigeons described in the preceding pages show that approximately 1.0 cc. of yeast filtrate given every other day is close to the amount required just to replace the deficiency of an exclusive diet of polished rice. Since this 1.0 cc. of yeast filtrate contains not exceeding 0.0018 gm. of vitamine, the daily vitamine requirement of pigeons must be somewhat less than 1 mg.

Recent observations have shown that pigeons given as much as they will eat consume about 30 gm. of food daily, per bird. If, as shown above, not more than 1 mg. of this must be vitamine, a properly balanced diet for a pigeon will be one which contains somewhat less than 0.0033 per cent of vitamine.

Although it cannot be stated with certainty, it is probable that a vitamine content of the same magnitude in the food of man would meet the requirements of the human organism.

SUMMARY.

a. In the case of pigeons, the vitamine deficiency of an exclusive diet of polished rice is just replaced by daily doses of 0.5 to 1.0 cc. of the clear filtrate from autolyzed brewers' yeast.

b. Doses of dried freshly pressed yeast approximately equivalent to 1.0 cc. doses of autolyzed yeast do not effectively replace the vitamine deficiency of a diet of polished rice.

c. Of two samples of dried yeast, one of which was autolyzed previous to being dried and the other not, the pigeons receiving the latter lost weight on a polished rice diet considerably more promptly than those receiving the former. The autolytic process therefore appears to influence favorably the activity of the vitamine of brewers' yeast.

d. On the assumption that all the nitrogen contained in fullers' earth which has been shaken with autolyzed yeast filtrate is derived from vitamine, the maximum quantity of the latter which can be present in the original yeast filtrate is 0.18 gm. per 100 cc.

e. The daily vitamine requirement of a grown pigeon is somewhat less than 1 mg. A diet containing 0.0033 per cent of vitamine, given in quantities ordinarily consumed by pigeons, will supply this requirement.

AN IMPROVED NEPHELOMETER-COLORIMETER.*

By PHILIP ADOLPH KOBER.

(From the Division of Laboratories and Research, New York State Department of Health, Albany.)

(Received for publication, January 4, 1917.)

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INTRODUCTION.

In biological chemical research work it is sometimes necessary to build a special apparatus or improve or modify an old one. At the outbreak of the European war it was impossible to obtain Duboscq colorimeters, and since it was desirable to remedy some defects in its design, a new instrument was developed and built in this country.[1] The purpose of this paper is to discuss some essential points of nephelometers and colorimeters, and to show still further improvements in the design and construction of a nephelometer-colorimeter.[2]

* A preliminary note was read at the New York meeting of the American Chemical Society, before the Biological Section, September 29, 1916.

[1] Manufactured by Lenz and Naumann, Inc., New York.

[2] The new instrument is manufactured by Klett Manufacturing Company, Inc., 202 East 46th Street, New York, and may be obtained from them or through dealers. The price, including two cups of any kind, is about $54.00. The lamp house is quoted at $24.00; extra cups at $1.50 each; light filters at $3.00 a pair. The whole outfit, including eight cups (two of each kind), mounted on a suitable black board with light switch, ready for use, is priced at $90.00.

Discussion of Other Instruments.

Bloor Nephelometer.—In order to provide a suitable mechanical and optical equipment for the Richards type of instrument, Bloor[3] modified the Duboscq colorimeter by removing the plungers and adding a few simple and easily constructed parts. That this resulted in a decided improvement for that type of instrument cannot be doubted, but the original criticisms[4] against the Richards instrument are still valid; *i.e.*, sources of error due to variations in the meniscus and in the indirect reflection of light from suspended matter in the lower part of the tubes. Bloor's objections to the plunger type of nephelometer are, however, no longer valid. As mentioned elsewhere in this paper, the plunger is made of one piece of glass by fusion of black glass tubing with an optical glass end, and therefore has no paint which may dissolve or peel off. It can now be kept as free from contamination as any other glass object and if necessary can be easily removed and replaced or cleaned with the strongest acid cleaning solution, if so desired.

An apparent advantage of the Richards type is that the solutions are not stirred or agitated by the plungers while obtaining a photometric balance, if we assume that agitation tends to agglutinate the suspensions, and ignore the fact that agitation also tends to keep any agglutinated particles in the suspension from settling. That this advantage is more apparent than real is due not only to the fact that all liquids are considerably more agitated, and practically to the same extent no matter what instrument is used, when adding the suspensions to the nephelometric containers, but also to the fact that the agitation produced by moving a plunger slowly up and down while making a reading is so slight that it must be negligible in most cases. However, any advantage derived by freedom from agitation is wholly lost by sources of errors, introduced in this instrument elsewhere as pointed out above. The relative accuracy obtained with it when compared with other instruments under favorable and equal conditions is not known.

Marshall-Banks Nephelometer.[5]—This instrument is described as having "equal columns of suspensions," and "actually measuring the reflected lights with a suitable photometer," "the photometric part of the apparatus consists of a wedge[6] of neutral tinted glass," while the "optical arrangement for observing the two beams of light" consisted of "a simple

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[3] Bloor, W. R., *J. Biol. Chem.*, 1915, xxii, 145.

[4] Kober, P. A., *J. Biol. Chem.*, 1912–13, xiii, 485.

[5] Marshall, J. T. W., and Banks, H. W., 3rd, *Proc. Am. Phil. Soc.*, 1915, liv, 180.

[6] The smoked glass wedge was used for photometric work in astronomy in 1882 (see Pickering, E. C., Nature, 1882, xxvi, 259) but was not adopted by photometrists.

arrangement of mirrors[7] by which a field far more sensitive than that of a Duboscq colorimeter may be obtained." As a detailed discussion of this instrument may appear in a separate communication on the chemical work also reported by Marshall and Banks in the papers dealing with their instrument, nothing further will be added here.

Duboscq Colorimeter.—Folin[8] mentions a number of defects in the Duboscq colorimeter: (1) inaccuracy of the zero points; (2) liability to dust interference; (3) inequality of illumination in the fields; (4) another disadvantage develops when the variations in the amount of color in the liquids to be measured are great, in which case two different and expensive colorimeters are necessary; (5) as the Duboscq cups have cemented bottoms they cannot be used for all solvents, and, as an investigator recently found, it was necessary to change the cement for certain work; (6) when the colorimeter is to be adapted as a nephelometer other disadvantages become apparent, especially in connection with the plungers and cups. This was pointed out in a previous paper.[17]

Lenzman-Kober Nephelometer-Colorimeter.[9]—This instrument practically eliminates the defects mentioned in the Duboscq colorimeter, with exception of defect (3), inequality of field illumination. This is however removed by means of the adjustable reflectors of the new lamp house (page 162). (1) In the Lenzman-Kober instrument one or two adjustments of the adjustable verniers eliminate any inaccuracy of the zero point; (2) no open spaces are to be found between the top of the eye piece and the liquids in the cups so that dust is excluded; (3) the scale and instrument are also so constructed that any height of liquid up to 110 mm. can be measured; (4) the colorimetric cups (Fig. 1) in this instrument, like the new nephelometer cups (Fig. 2) with which they are interchangeable, are fused instead of cemented and are therefore of one piece of glass, usable for all solvents. (5) The black glass plunger[10] (Fig. 3) with fused-

[7] A similar arrangement of mirrors is used in the Donnan-Köhler colorimeter (described in *Abderhalden's Handb. biochem. Arbeitsmethoden*, 1910, i, 648), which arrangement, owing to the loss of light by reflection of the glass of the front mirror, and owing to the unequal paths the light from the cups must travel, requires, as stated in the book, an empirical correction.

[8] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 484.

[9] Made by Lenz and Naumann, Inc., New York, at the suggestion and with the cooperation of the author.

[10] For this idea and for much experimental work at his own expense, I am indebted to Mr. Hugo Schnackenberg, of the firm of Potter and Schnackenberg, Manufacturing Opticians, New York.

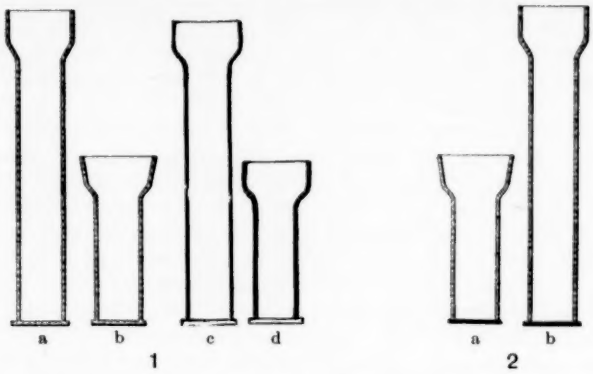

FIG. 1. Colorimetric cups. (*a*, *b*) Transparent and optically clear bottoms fused to the sides; (*c*, *d*) black glass sides which eliminate the colorimetric light shield.

FIG. 2. Nephelometric cups. (*a*, *b*) Black glass bottoms fused to transparent sides. Diameter of cups just ½ mm. larger than that of the plungers.

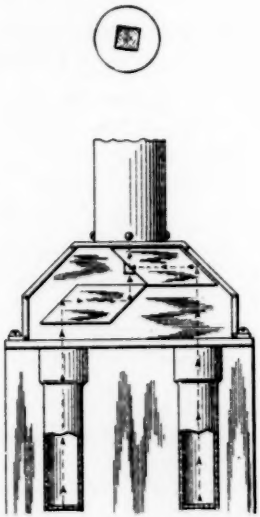

FIG. 3. Prism arrangement and plungers. Black glass plungers with optically clear glass ends, fused together. Prism arrangement, which gives a field similar to that of Lummer-Brodhun.

in, optically clear bottoms, not cemented, as they might seem and a few have believed them to be, makes its use as a colorimeter and nephelometer a matter of equal ease and accuracy, entirely eliminating the use of asphaltum paint for this purpose. The best Duboscq plungers show a distinct color or absorption of light when compared with these new plungers. (6) The new design of a field (Fig. 3) similiar to that of Lummer-Brodhun, a square within a circle, makes its use, when correctly made and adjusted, much more comfortable for the eye and more sensitive.

Unfortunately the first lot of twenty-five instruments, owing to the difficulties arising from the scarcity of good optical glass, superimposed upon labor difficulties, were imperfectly and crudely made, poorly if at all adjusted, so that the advantages of the new design were rarely apparent. In the second lot of twenty-five and subsequent instruments, the difficulties seem to have been completely overcome.

This instrument for its use as a nephelometer, however, had a disadvantage in that it used automatic black cloth curtains necessary to insure a black background for the opalescent solutions. In common with the Duboscq colorimeter, it had also racks and pinions for raising the cups or plungers, which are difficult to make and almost impossible to use without having appreciable lost motion.

The New Instrument.

The Nephelometer-Colorimeter.—By using a screw arrangement,[11] as shown in Figs. 4 and 5, both the black curtains and this lost motion can be eliminated and the construction considerably simplified. As may be seen, it also has all the advantages of the Lenzman-Kober instrument mentioned before. Furthermore, with the double milled head, a double adjustment is practically secured: (1) with the small diameter, a quick action for rough adjustment; (2) with the large diameter, a slow action, for fine adjustment. Another advantage in construction is seen in the scale, which shows that it is removable, so

[11] For this idea and for much experimental work at his own expense, I am indebted to Mr. R. E. Klett, of the Klett Manufacturing Company, Inc., New York.

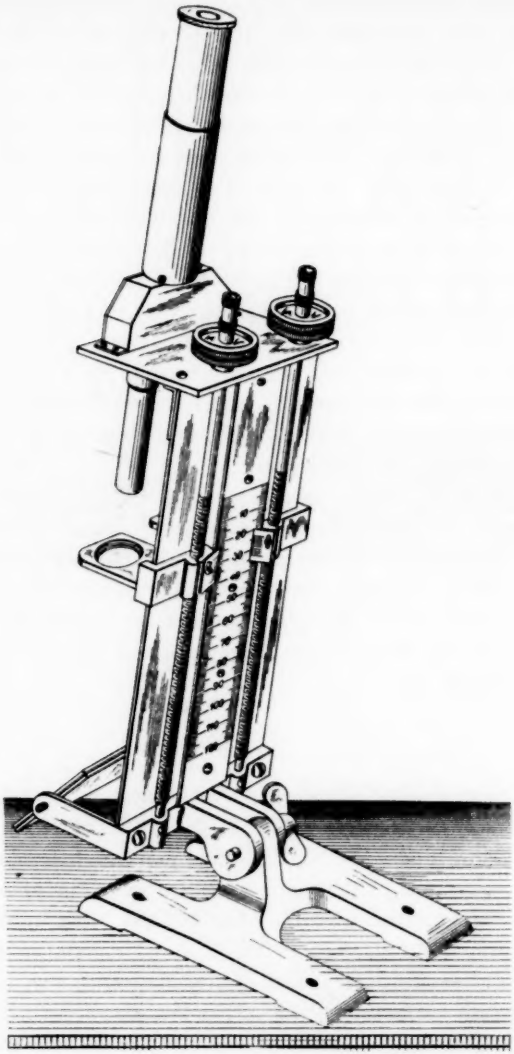

FIG. 4. The new nephelometer-colorimeter, showing screw arrangement with adjustable verniers, also the double milled head. There are no open spaces through which dust may enter and light escape when fitted to the lamp house (see Fig. 5).

that it can be repaired or replaced by a new one when necessary. As these scales are made with a dividing machine, they are usually very exact.

The Lamp House.—In a previous communication[12] the theoretical qualifications of a nephelometric light were discussed. The lack of a convenient source of low voltage (from 6 to 20

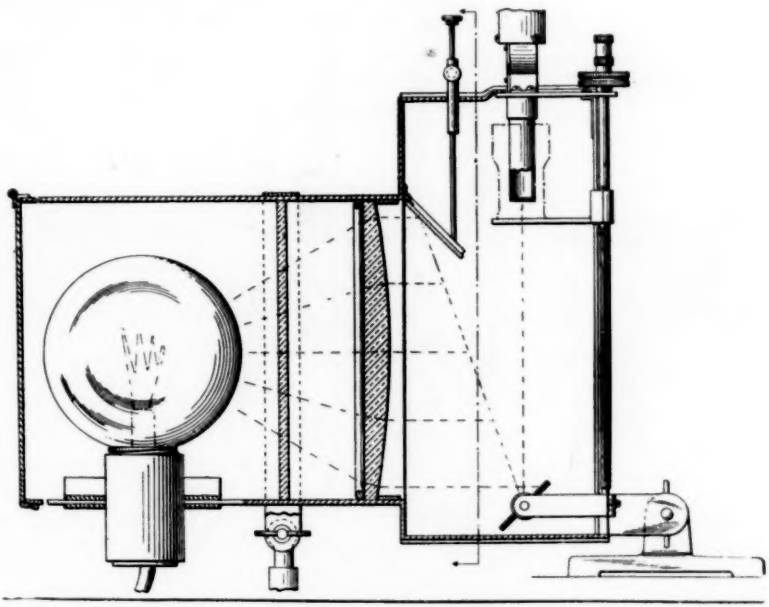

FIG. 5. Lamp house and instrument, showing the concentrated filament lamp, air space, condenser, and lamp house. When the doors (not shown) are closed, no light is visible except in the eye piece. The inclined angle of the instrument, which allows air bubbles to escape from underneath the plungers, and the exact position and angle of reflectors are not shown in the sketch.

volts) makes it still difficult if not impossible to obtain parallel rays of light, without using an arc lamp. The arrangement, as shown in Fig. 5, of a concentrated tungsten filament 110–120

[12] Kober, P. A., and Graves, S. S., *J. Ind. and Eng. Chem.*, 1915, vii, 843.

volt lamp[13] (stereopticon) with a condenser gives a very strong light, suitable for most work. A pane of glass is interposed between it and the condenser, so as to provide an air space, to prevent unnecessary heating of condenser and nephelometer. The lamp house can then be directly connected to most lighting circuits. The reflectors (Fig. 6) of the lamp house are made in pairs, so that the light which falls on each side of the colorimeter

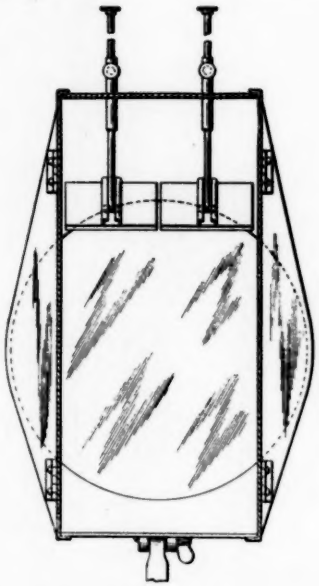

FIG. 6. Colorimetric reflectors, showing two separate reflectors by means of which the light reaching the eye piece may be adjusted to equality.

can be adjusted to produce equality in the field, thus eliminating a defect in the Duboscq colorimeter mentioned by Folin. This defect, which is more or less present in all these instruments, is due to the fact that it is impossible to make optical and mechanical parts perfectly.[14] The individual reflectors once adjusted

[13] McDermott, F. A., *J. Am. Chem. Soc.*, 1914, xxxvi, 454, describes a lamp of the same construction.

[14] In one of the most accurate of physical instruments, the balance, equality of sides, or the zero point, often changes and is also not adjusted until it is set up ready for use.

and the position marked on the stem, they can be removed from the field of light for nephelometric work and replaced if necessary in a moment. In Fig. 7 is shown a removable light filter, which can be made of this so called "daylight" glass if its absorption of light is not too great, or a light blue glass to remove any yellow in the artificial light, so as to measure any yellowish liquids. This allows the instrument to be set up perma-

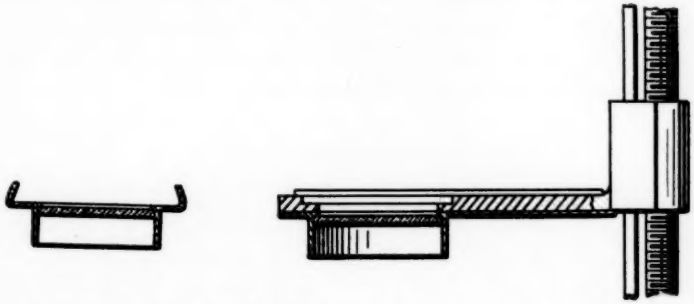

FIG. 7. Removable light filters. These filters, being attached underneath the cup holders, may remain on the instrument without interfering with nephelometric work.

FIG. 8. Eye support which steadies the eye and keeps it at one point.

nently, screwed to a base, so as to be portable, and yet equipped to measure quantitatively colored and opalescent liquids with equal facility and almost equal accuracy. There is a growing conviction that in general it is easier to measure nephelometrically than colorimetrically, due to the fact that the more light absorbed by the solution, *i.e.*, the more it approaches uniform absorption, the easier it is to measure. For example, a blue or a

green[15] tint is easier to match than a yellow tint. In Fig. 8 is shown an eye support which will be of assistance in keeping the eye steady and always at the same point, an important requisite for precision work.

Results with the New Instrument.

The following readings made on the first factory model, by an assistant who had practically no photometric experience, show not only the sensitiveness of the instrument, but also the accuracy obtainable in nephelometric work.

Casein with Sulfosalicylic Acid.

Solution I.	Solution II.	Solution III.	Solution III.
16.3	24.9	30.3	30.2
16.5	24.6	30.5	30.4
16.2	24.7	30.2	30.3
16.3	24.5	30.3	30.4
16.33	24.67	30.33	30.33

As the new instrument has stationary plungers and movable cups, while most of the previous nephelometric work was done with movable plungers and stationary cups, it was important to run a nephelometric curve to see the effect on the constants. The curve shown in Fig. 9, with casein and sulfosalicylic acid, shows that in these new instruments the nephelometric constant has a new value and sign.

[15] Elbert, *Wiedemann's Ann.*, xxxiii, 136, found the following relative values of stimulus threshold for one individual.

	Wave length. μ	Threshold values.
Red	0.675	0.7
Yellow	0.590	2.2
Green	0.530	0.5
Bluish green	0.500	1.0
Blue	0.470	7.0

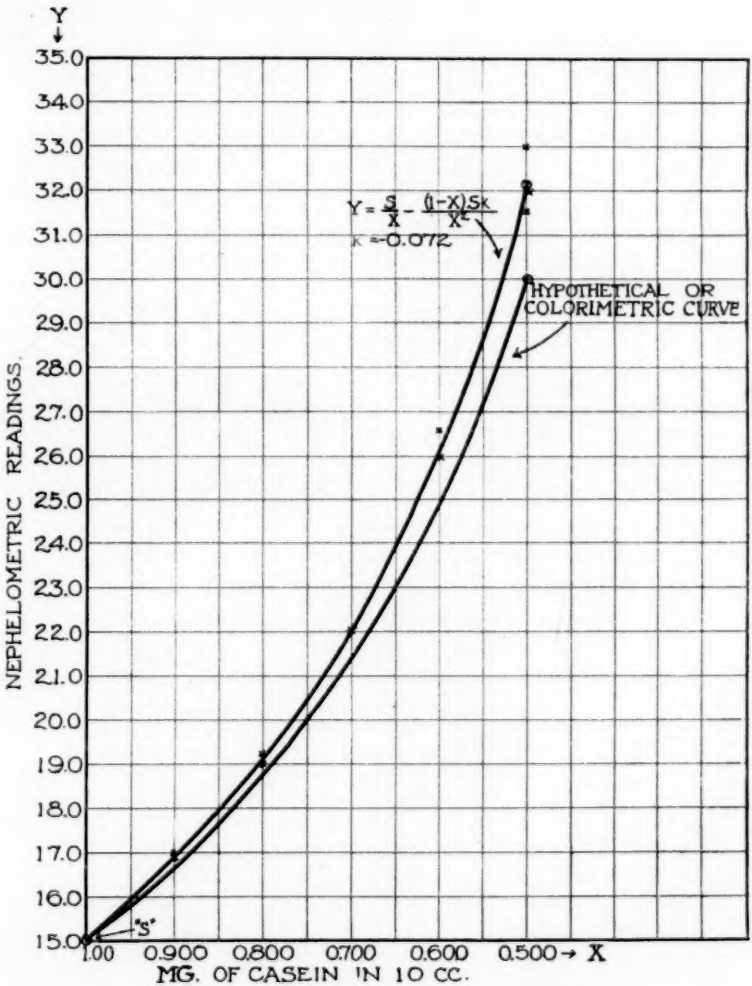

FIG. 9. Curve, with the new instrument, using casein.

Ratio of solutions. x	Actual readings.	Average readings. y	$y = \frac{s}{x} - \frac{(1-x)\,s\,k}{x^2}$ $k = -0.072$
	mm.	*mm.*	
1.0	15.0 15.0 $= s$	15.00	15.00
0.9	16.9 17.0 16.8	16.90	16.80
0.8	18.9 18.9 19.2	19.00	19.09
0.7	22.00 22.00	22.00	22.07
0.6	26.0 26.5	26.25	26.20
0.5	31.50 32.0 33.0*	32.17	32.16

* This curve was made quickly at the close of a day (after 9.30 p.m.) and the last readings show the effect of eye fatigue.

With casein, using the old type of instrument where the light from the weaker solutions had to travel a mean distance that was shorter than the mean path of the stronger solution, the constant was about + 0.20, whereas in this new instrument where the light from the stronger solution has shorter mean distance to travel, the constant is − 0.072.

From this we learn that part of the deviation from the hypothetical or colorimetric curve was due to differences in the mean paths, while the other part of the deviation must be due to the effect of the suspension on the amount of light transmitted.[16]

While it is to be remembered that this formula expresses only the ratio of the solutions and is not for absolute photometry, it is, nevertheless, remarkable that so simple and empirical a formula is capable of expressing with precision a phenomenon which has so many factors.

With the same instrument as a colorimeter, measuring cupric sulfate and potassium dichromate solutions, the following readings were obtained.

$CuSO_4$	$K_2Cr_2O_7$
21.0	19.9
20.8	19.5
20.7	19.6
20.8	19.6
21.1	19.9
Average 20.9	19.7

[16] Discussion of this and other aspects of nephelometry is reserved for a subsequent publication. It may be possible to obtain a formula having a constant, independent of the type of instrument.

Directions for Using the Instrument.

In previous papers[17] the use of nephelometric curves (Fig. 9) and a formula was discussed and it was pointed out that it is necessary to standardize the instrument, using solutions of different concentrations. When the instrument changed so that a restandardization was necessary, a nephelometric formula obviated considerable work, especially the readings for and the drawing of a new curve.

Since the formula is complicated and many prefer to do without mathematical calculations, the following scheme can be used. The instrument is standardized as before, but the curve (see Fig. 9) is used alone in getting the amount of substance equivalent to the readings. When the value of the standard readings (designated by s in the formula) changes, the height of the solution used as a tare is adjusted so that the original reading for s is obtained, and therefore the original curve is applicable. It is equivalent to changing the zero point of a balance by adjustment so as to avoid calculations.

It is doubtful, however, whether the use of such a curve can ever be as accurate as use of the formula, owing to the fact that the accuracy with which one can draw and use a curve is decidedly limited, while the accuracy with which one can express relationships by means of an equation is unlimited.

A number of inquiries have been received as to obtaining the correct zero reading with the verniers, when the cups are too long to permit the plunger to touch the bottom of the cups, *i.e.*, to obtain the exact position on the scale for zero light or color. A number of methods could be suggested but the following is probably the simplest. A piece of wood, such as an ordinary cylindrical or hexagonal lead pencil or a thick glass rod or tube, about the thickness of the plunger, is cut off about 70 mm. long, making sure that the ends are parallel and at right angles to the sides. The length of this is then accurately measured with the scale on the instrument, or any other accurate mm. scale. This meas-

[17] Kober, *J. Biol. Chem.*, 1912–13, xiii, 485. Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2377 (also given in Hawk, P. B., Practical Physiological Chemistry, Philadelphia, 5th edition, 1916, 294).

ured rod[18] is put into the cup and the instrument adjusted until the plunger touches it, or is flush with its optical end. If the screw of the vernier is loosened and the vernier set on scale to correspond with the length of the rod then the vernier and scale will give the actual height of space or liquid underneath the plunger in actual work. It is not safe to assume that all the cups have bottoms of the same thickness.

SUMMARY.

1. A new nephelometer-colorimeter has been described which has the following advantages: (*a*) a screw arrangement for changing the heights of liquids, and therefore the elimination of lost motion inherent in racks and pinions; (*b*) the elimination of dark cloth curtains; (*c*) black one piece glass plungers; (*d*) fused one piece nephelometric and colorimetric cups; (*e*) a convenient eye support.

2. A simple and convenient lamp and lamp house are described, having adjustable reflectors which permit the amount of light reaching both sides of the colorimetric field to be adjusted to equality.

3. A new simplified method of using the instrument is given.

[18] An accurately measured aluminium rod called "zero-gauge" can be obtained from Klett Manufacturing Company, Inc., New York.

A RAPID METHOD FOR DETERMINING CALCIUM IN BLOOD AND MILK.

By HENRY LYMAN.

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(Received for publication, January 4, 1917.)

In 1915[1] I published a short turbidity method for determining calcium in feces and urine, which consisted briefly of the following steps. The calcium was precipitated as calcium oxalate in the urine (or the acid extract of fecal ash) under conditions to minimize the occlusion of magnesium; the precipitate was centrifuged, washed, dissolved in dilute hydrochloric acid, and treated with an aqueous solution of the potassium soap of castor oil. The resulting cloud of calcium soap was read on the Duboscq colorimeter against a standard solution of calcium oxalate in hydrochloric acid, treated with the soap at the same time as the unknown.

I have now succeeded in applying essentially these same principles to the determination of calcium in small amounts of blood and of milk, the two chief differences being, first, that since the soap cloud produced by the very small quantity of calcium available in, say, 5 cc. of blood is too faint to be read on the colorimeter, the nephelometer is used instead, and, second, that a solution of ammonium stearate is substituted for the castor oil soap. The method, as developed, is short, taking 2 hours for a set of four determinations as against 3 full days by the ashing and gravimetric procedure; it requires but 5 cc. of blood instead of more than 300 cc.; only two instruments are used, the nephelometer and the centrifuge; the reagents employed are easily obtainable, an important factor at the present time; and finally, the results, as will be shown, are accurate to within less than 1 per cent.

[1] Lyman, H., *J. Biol. Chem.*, 1915, xxi, 551.

PROCEDURE.

For Blood.—To draw the blood, a paraffined pipette attached to a hollow needle by a piece of rubber tubing (according to the method of Folin and Denis[2]) is employed. The potassium oxalate is naturally omitted. Run 5 cc. of blood into a small flask containing 15 cc. of trichloroacetic acid, 6.5 per cent, while agitating the flask. Mix and let stand for a few minutes. Filter through a folded calcium-free filter paper. Pipette 10 cc. of the filtrate into an Erlenmeyer flask of about 50 cc. capacity. Add one drop of methyl orange, 0.1 per cent. Add 2 N ammonium hydrate drop by drop until just yellow. Add nitric acid, 0.05 N, dropwise until pink, and then 1 cc. more. Add 1 cc. of oxalic acid, 4 per cent. Add 1 cc. of sodium acetate, 20 per cent, dropwise. Cool under the water tap until a faint cloud appears Shake 10 minutes or stand over night at room temperature, as convenient. Rinse the stopper with a few drops of ammonium oxalate, 0.5 per cent. Pour into a centrifuge tube and centrifuge. Pipette off supernatant liquor. Rinse the flask with 5 cc. of ammonium oxalate, 0.5 per cent, pour into centrifuge tube, stir, rinsing the rod with 0.5 per cent ammonium oxalate, and again centrifuge. Pipette off supernatant liquor. Dissolve precipitate in 5 cc. of 0.1 N nitric acid by means of stirring, and pour into original flask. Agitate a moment to dissolve any precipitate adhering to the walls. Rinse the rod and centrifuge tube with 5 cc. of water, and pour into the flask.

Into another flask of about 100 cc. capacity, pipette 20 cc. of the standard calcium oxalate solution. Pipette 50 cc. and 25 cc. respectively of the ammonium stearate reagent into two clean dry beakers. Pour the standard solution into the 50 cc. of reagent, and the unknown into the 25 cc., and pour back twice. Stopper and let stand 10 minutes. Fill both nephelometer tubes with the standard, set the left side at 32 mm_v and take a careful reading to be sure that the two sides of the instrument are balanced. Replace the standard on the left with the unknown and read. Care should be taken before reading to remove with a glass rod any bubbles adhering to the walls of the nephelometer tubes.

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

Calculation.

If the unknown is set at 32 mm. and the standard is read, the reading divided by 4 will equal the number of mg. of calcium in 100 cc. of blood. Note that calcium is calculated as calcium and not as the calcium oxide.

For Milk.—Pipette 10 cc. of the well mixed sample into a volumetric flask, a 200 cc. flask for cow's milk or a 100 cc. flask for human milk. Make up to volume with distilled water and mix thoroughly. The mixture is now of approximately the same per cent calcium content as blood. Pipette 5 cc. of this mixture into 15 cc. of trichloroacetic acid, 6.5 per cent, let stand, filter, and proceed as with blood. To find the number of mg. of calcium per 100 cc. of milk, the nephelometer reading is multiplied by 5 or by 2.5, according as the milk was originally diluted to 200 or 100.

Reagents, Etc.

Trichloroacetic Acid Solution, 6.5 Per Cent.—This reagent, suggested by Greenwald,[3] is used for the coagulation of both blood and milk, three volumes of the reagent being taken for one of the substance to be coagulated. It gives a water clear filtrate, free from protein, and, being highly dissociated, holds in solution any calcium salts present. It, as well as all other materials, should be tested for calcium before use.

Filter Paper.—As the filter paper used for common laboratory purposes contains considerable calcium, a paper must be selected for filtering the coagulated blood or milk which has been washed in acid. For this purpose Baker and Adamson's paper "A" washed in hydrochloric and hydrofluoric acids was employed For filtering the reagents absorbent cotton washed first with hydrochloric acid, 10 per cent, then with water, until the wash water is no longer acid to litmus, and finally dried, may be used.

Indicator, Methyl Orange, 0.1 Per cent.—Dissolve 0.1 gm. of methyl orange in 10 cc. of alcohol and make up to 100 cc. with water.

Nitric Acid, 0.1 N *and 0.05* N.—The concentrations need not be exact, provided the same strength of acid is used in all steps

[3] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

of the process, including the making up of the standard. For convenience a stock solution of 2 N may be prepared and the lower concentrations made up from this as needed.

Ammonium Hydrate, 2 N.—This need not be accurate. 13.5 cc. of ammonium hydrate, sp. gr. 0.9, made up to 100 cc., with water will serve the purpose.

Oxalic Acid, 4 Per Cent.—As 1 cc. of this solution is used to precipitate the calcium in only 2.5 cc. of blood, it will easily be seen that the excess over the theory is very large. Unless such an excess is present, however, precipitation does not begin promptly, owing to the low concentration of calcium oxalate.

Sodium Acetate, 20 Per Cent.—20 gm. of crystallized sodium acetate dissolved in 100 cc. of water.

Ammonium Oxalate, 0.5 Per Cent.

Ammonium Stearate Reagent.—Dissolve 4 gm. of stearic acid and 0.5 cc. of oleic acid in 400 cc: of hot alcohol. Add 20 gm. of ammonium carbonate dissolved in 100 cc. of hot water and allow the mixture to boil for a few moments. Cool. Add 400 cc. of alcohol, 100 cc. of water, and 2 cc. of ammonium hydroxide (sp. gr. 0.9). Filter. This solution should be as clear as freshly distilled water and perfectly colorless. If well stoppered it keeps indefinitely. Before using for analysis, test as follows: Into two flasks pipette respectively 10 and 5 cc. of the calcium oxalate standard and to the 5 cc. add 5 cc. of nitric acid, 0.05 N. Treat both with 25 cc. of the ammonium stearate reagent and read on the nephelometer. If they do not read exactly 2 to 1 there is some impurity present in the chemicals used. The alcohol—if, as is usual in laboratories, it has stood in a wooden barrel—will give a yellow coloration with ammonia and will contain suspended particles which reflect light in the nephelometer. It should be redistilled with a little calcium carbonate. Stearic acid may be purified by recrystallizing from boiling alcohol. Ammonium carbonate may be resublimed.

Calcium Oxalate Standard, 10 Cc. to Contain 0.2 Mg. of Calcium in 0.05 N HNO_3.—Dissolve 72.9 mg. of pure calcium oxalate ($CaC_2O_4 + 1H_2O$) in 25 cc. of nitric acid, 2 N, and make up to 1,000 cc. with water. Since the presence of chlorides affects the solubilities of calcium soaps, nitric acid is used as a solvent throughout instead of hydrochloric.

Accuracy of the Method.

The method was checked as follows: (1) 5 cc. portions of the calcium oxalate standard (equivalent to 0.1 mg. of calcium) were run through the described procedure, including the preliminary treatment with trichloroacetic acid solution. Since half the trichloroacetic mixture is taken, only 0.05 mg. of calcium was being determined at the end. The nephelometer readings against 10 cc. of the standard (0.2 mg. of calcium) were exactly 8:32, giving a calcium content for the solution of 2 mg. per 100 cc., which was correct. No calcium was therefore lost and, since all solutions and materials had been carefully tested for calcium, none had been added. Next, a solution of calcium chloride was made up and a portion analyzed by the gravimetric method. From the data thus obtained the solution was then diluted to contain 5 mg. of calcium per 100 cc., and two 5 cc. portions were run through the method. Respectively 4.96 and 4.94 mg. per 100 cc. were recovered.

(2) Samples of blood taken from the same animal, as nearly as possible at the same time, were ashed and done by the new method. No attempt was made to perform the gravimetric procedure. Such large amounts of blood, at least 300 to 500 cc., must be handled that the loss by spattering and fusing renders this method less accurate than the new one which it is being used to check.

Portions of blood were drawn from the living animal into paraffined pipettes, the first sample being run into a platinum dish for ashing, the second into trichloroacetic acid solution for treatment by the new method, the third into platinum, etc. The samples in platinum were then dried over night on a hot plate and cautiously ashed by hand over a Bunsen burner. The residue was taken up with nitric acid, 0.1 N, and the calcium precipitated as oxalate, washed, treated with stearate solution, and read against the standard on the nephelometer. Much trouble was experienced with this mode of ashing. In the first place the results were not concordant and in the second the values were almost always below (never above) the values obtained on the same blood by the new method. There are three obvious sources of error: (*a*) If an oxidizing mixture, such as

nitric and sulfuric acid, is employed there is invariably a fine spray thrown off at the moment when the ash becomes dry, as may be shown by placing a metal plate about the crucible or dish. This same objection holds when successive small portions of water are used, as is sometimes done. (*b*) If no oxidizing agent is present it is very difficult to burn the residue free from carbon, and if this is not done completely the blood charcoal left has such strong adsorptive power as to make it impossible to extract the calcium salts by simple treatment with dilute mineral acid. (*c*) If much heat is employed the residue fuses in a thin layer over the surface of the dish and can only be removed by mechanical means; *e.g.*, the usual scrubbing with sand. Table I, taken from five consecutive experiments, will serve as an illustration.

TABLE I.

Calcium Recovered per 100 Cc. of Blood.

Animal No.	New method.	Ashing.
	mg.	*mg.*
Cat 1	5.9	4.6
	5.9	4.0
" 2	5.83	
	5.82	5.78
	5.83	
" 3	5.82	5.84
	5.81	4.88
Man	6.12	5.9
		5.6
Cat 4	6.16	4.56

At the suggestion of Dr. R. D. Bell, ashing was next attempted by means of the hot air bath alone, without use of the free flame, and this proved perfectly successful. Samples of blood in platinum, taken as before, were first dried in a bomb furnace at about 200°C. for 6 or 7 hours. The temperature was then gradually raised to the full heat of the furnace, 320°C., and maintained there for 4 hours, by which time most of the tarry bodies had been

distilled off. Next the dishes were transferred to a muffle and the muffle was kept at a dull red heat for 4 hours more, the dishes being supported on small tripods to prevent their touching the walls or floor of the muffle. The residue, a reddish powder, was then taken up with nitric acid, 0.1 N, and run through the method as before. Table II gives the result of six consecutive experiments.

TABLE II.

Calcium Recovered per 100 Cc. of Blood.

Animal No.	New method.	Ashing.
	mg.	*mg.*
Cat 1	6.71	6.73
" 2	7.62	7.60
Rabbit	8.37	8.37
Man (1)	6.13	6.13
" (2)	6.06	6.14
" (3)	5.54	5.52

Tests for iron, after the calcium had been thrown down as oxalate and the precipitate washed, were uniformly negative.

One possible source of error then remained to be examined. It is conceivable that the trichloroacetic acid might dissolve some protein-like substance from the blood, which could be carried through the various precipitations and finally thrown down by the large excess of ammonia present in the ammonium stearate reagent. This might offset a possible loss of calcium, although, of course, the probability of the two errors so exactly balancing each other is slight. If, however, such a hypothetical protein body were destroyed by ashing the filtrate from the trichloroacetic acid precipitation, a determination of calcium in this ash would yield lower figures than where the proposed method was carried straight through on another sample of the same blood. That such is not the case is shown in Table III.

(3) The milk determinations were checked up exactly as with blood. After the samples had been diluted as indicated (p. 171), 5 cc. portions were pipetted into platinum dishes for ashing and into trichloroacetic acid solution for determination by the new method. Since, however, the residue from such small amounts

TABLE III.

Calcium Recovered per 100 Cc. of Blood.

Animal No.	New method.	Ashing.
	mg.	*mg.*
Cat 1	7.10	7.12
" 2	5.82	5.86
Rabbit 1	5.84	5.80
" 2	8.43	.44
Man	6.22	6.20

of milk could be readily burned, the free flame was employed instead of the hot air bath. Table IV gives the result of seven such experiments on milk from different sources. It may be remarked that Sample 2, which shows an unusually low calcium content, came from a somewhat questionable source and possibly had been diluted.

TABLE IV.

Calcium Recovered per 100 Cc. of Milk.

Sample.	New method.	Ashing.
	mg.	*mg.*
1	99.6	99.3
2	86.6	86.6
3	127.5	127.0
4	121.5	121.5
5	122.5	123.5
6	129.5	130.0
7	127.0	127.5

DISCUSSION.

When the work was first begun on this method it was thought that by using a large excess of potassium oxalate it might be possible to precipitate the calcium in both blood and milk directly, thus avoiding the steps of coagulation and filtering. The results were, however, uniformly too low in both cases. In blood either part of the calcium is in chemical combination, perhaps with a protein, or else the blood proteins act as a protective colloid, thus preventing the calcium oxalate from coming down.

This is shown by the following experiment. A wide-mouthed bottle, holding 2,000 cc. and containing 20 gm. of finely powdered potassium oxalate, was filled with ox blood, directly from the vein of the animal, and thoroughly shaken. It was then left in the ice box at 3°C., for 3 days, again shaken, and a portion centrifuged. 50 cc. of the top fluid, which should have been calcium-free, were then run into 6.5 per cent trichloroacetic acid solution, allowed to stand a short time, and filtered. 150 cc. of the filtrate were evaporated to dryness in a large platinum dish and the residue was cautiously burned. The ash was taken up with 0.1 N nitric acid and the calcium in this solution determined by the new method. 0.60 mg. of calcium per 100 cc. of blood was recovered, or a little over 10 per cent of the original calcium content of the blood. The blood of a cat treated in the same manner yielded 1.87 mg. of calcium per 100 cc. of blood, or 31 per cent. A considerable amount of calcium had therefore escaped precipitation as oxalate.

In milk, as has been shown by Van Slyke and Bosworth,[4] the calcium is partly in true solution, partly in a colloidal state, but chiefly combined with protein, probably as calcium caseinate. This being the case, the low results obtained were what might have been expected. Attempts at direct precipitation were therefore abandoned, and, on the suggestion of Professor Folin, the trichloroacetic acid coagulation was adopted instead. Apparently this treatment is sufficient to liberate the calcium from its caseinate in milk and from its protein combination in blood, as well as to hold the inorganic salts in solution. In the precipitation of calcium oxalate from its acid solution McCrudden's[5] technique has been followed, modified of course for the low concentration here met with. This manipulation was based on the principles laid down by Richards[6] and is now too well known to need comment. Fortunately the great insolubility of calcium oxalate in dilute solutions of ammonium oxalate permits very small quantities of this salt to be handled without perceptible loss.

[4] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 135.

[5] McCrudden, F. H., *J. Biol. Chem.*, 1911, x, 187.

[6] Richards, T. W., McCaffrey, C. C., and Bisbee, H., *Proc. Am. Acad. Arts and Sc.*, 1901, xxxvi, 377.

The development of the ammonium stearate reagent involved several principles worthy of mention. In the first place, as the calcium soap of castor oil is slightly soluble in the large excess of alkali necessary to hold even the potassium soap in solution, the clouds produced by small amounts of calcium were not quantitative and it became necessary to find a soap of lower solubility. After trial of palmitates, oleates, and some others without success, it was found that calcium stearate would answer the purpose, provided a solution of some salt of stearic acid could be prepared which would remain perfectly clear for a reasonable length of time. Commercially, three agents are commonly employed in the manufacture of liquid soaps, to keep the solution from hydrolyzing: first, excess of alkali, preferably carbonate; second, alcohol; and third, oleates, which have the remarkable power of restraining the hydrolysis of other soaps. The insolubility of ammonium stearate is such that it was found necessary to use all three of these, as may be seen by reference to the description of this reagent.

The large excess of stearate in proportion to the calcium to be precipitated is to be noticed. If this excess is not present, the calcium soap, instead of remaining suspended in an even cloud, settles rapidly to the bottom, either as a flocculent mass, or as a crystalline precipitate, and is of course worthless for nephelometric determination. Finally, the saponification of fats or oils rich in stearates by sodium or potassium hydroxide was found to yield an unsatisfactory reagent, and the method of direct union of stearic acid with ammonia in hot alcohol was selected instead. Aqueous ammonium hydrate is not suitable for this purpose.

In closing I should like to point out that as the introduction of 0.002 mg. of calcium in the course of the determinations would result in an error of 1 per cent, the materials used must be of the highest purity. It is well, occasionally, to run a sample of the standard through the whole procedure to be sure that there is neither gain nor loss at the end.

STUDIES ON ANIMAL DIASTASES.

I. THE INCREASED DIASTATIC ACTIVITY OF THE BLOOD IN DIABETES AND NEPHRITIS.

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Although it has been recognized since the time of Magendie[1] that blood possessed active diastatic properties, no studies of a conclusive nature appear to have been published relative to the activity of the blood diastase in human subjects suffering from diabetes. From an investigation of this question on human diabetics, Foster,[2] Bainbridge and Beddard,[3] Schlesinger,[4] Wynhausen,[5] Ghedini,[6] and Stocks,[7] were unable to come to any conclusions, though Loewi[8] regarded the diastase of considerable importance, and Moeckel and Rost[9] state, apparently from meager data, that the diastatic activity of the blood in human diabetes is almost always higher than normal.

The results obtained on depancreatized dogs have likewise been contradictory, although Milne and Peters,[10] in a comparatively large series of experiments carried out on the dog, uniformly obtained a decided increase in the diastase of the blood after removal of the pancreas. We have obtained similar results on one of Dr. Murlin's depancreatized dogs.[11]

Milne and Peters state: "The comparison of these results with what is found in diabetes is of interest, as it might well be that the excessive amount

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[1] Magendie, *Compt. rend. Acad.*, 1846, xxiii, 189.

[2] Foster, M., *J. Anat. and Physiol.*, 1867, i, 107.

[3] Bainbridge, F. A., and Beddard, A. P., *Biochem. J.*, 1907, ii, 89.

[4] Schlesinger, W., *Deutsch. med. Woch.*, 1908, xxxiv, 593.

[5] Wynhausen, O. J., *Berl. klin. Woch.*, 1910, xlvii, 1281.

[6] Ghedini, G., *Clin. med. ital.*, 1912, li, 146.

[7] Stocks, P., *Quart. J. Med.*, 1916, ix, 216.

[8] Loewi, O., *Marburger Sitzber.* (Nov.), through *Biochem. Zentr.*, 1905–06, iv, 271.

[9] Moeckel, K., and Rost, F., *Z. physiol. Chem.*, 1910, lxvii, 433.

[10] Milne, L. S., and Peters, H. LeB., *J. Med. Research*, 1912, xxvi, 415.

[11] Our thanks are due to Dr. Murlin for placing this material at our disposal.

of sugar in the blood is produced by an increased diastatic action of the serum causing an excessive conversion of glycogen into glucose." They do not appear, however, to have studied this question on cases of human diabetes. It is also worthy of note in this connection that Lesser[12] found that the postmortem glycogenolysis of the liver of depancreatized frogs was three times the normal.

It has been recognized for some time that nephritis may be accompanied by a decrease in the excretion of diastase in the urine, which fact has, indeed, been utilized as a means of determining the impairment in renal function.[13] That this decreased excretion of the diastatic enzyme is accompanied by an accumulation in the blood has been shown by Lœper and Ficaï,[14] Hirata,[15] and Stocks,[7] the last of whom has reported a number of observations with the Wohlgemuth method on cases of nephritis in human subjects.

The paucity of reliable data on the subject of the diastatic activity of the blood appears to be due to the lack of a sufficiently delicate and accurate method. Of the different methods employed, those dependent upon the estimation of the reducing sugars formed are the most satisfactory. The procedure introduced by Lewis and Benedict,[16] for the estimation of the sugar of the blood has been found useful in the estimation of the diastatic activity.

Method.

Two 2 cc. samples of oxalated blood are taken, one being employed as a control. The control tube[17] is made up to 10 cc. with distilled water, and the tube to be employed for the test to 9 cc. Both tubes (cylindrical centrifuge tubes) are now placed in a water bath at 40°C. As soon as the contents of the tubes have been brought to this temperature, 1 cc. of 1 per cent soluble starch is added to the second tube, the contents are mixed, and incubation is then carried out for exactly 15 minutes at 40°C. After

[12] Lesser, E. J., *Biochem. Z.*, 1913, lv, 355.

[13] See Geyelin, H. R., *Arch. Int. Med.*, 1914, xiii, 96.

[14] Lœper, M., and Ficaï, J., *Arch. méd. exp.*, 1907, xix, 722.

[15] Hirata, G., *Biochem. Z.*, 1910, xxviii, 23.

[16] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

[17] If a sugar estimation is being made simultaneously, there would appear to be no good reason for running a control, since a 15 minutes' incubation results in no appreciable change in the blood sugar. The control may, however, be made the basis of the blood sugar estimation.

the incubation has been completed, about 1 gm. of dry picric acid is at once added to each tube and the mixtures are stirred. When the proteins are precipitated, the tubes are centrifuged and the yellow supernatant fluid is filtered. The sugar in 3 cc. portions of the filtrates is now estimated according to the technique described by Myers and Bailey.[18] Correction is made for the sugar originally present in the blood (with the aid of the control) and for the slight reducing action of the soluble starch. The results have been recorded in terms of the percentage of the soluble starch (10 mg.) transformed to reducing sugars (calculated as glucose) by the 2 cc. of blood employed.

TABLE I.

The Influence of Varying the Amount of Substrate on the Diastatic Activity of 2 Cc. of Blood.

Case.	Blood sugar.	Amount of soluble starch employed.			
		10 mg.	20 mg.	30 mg.	40 mg.
		Reducing sugar formed.			
	per cent	*mg.*	*mg.*	*mg.*	*mg.*
N. L.	0.50	4.65	4.66	4.63	4.46
L. O.	0.46	4.57	4.36	4.75	4.54
G. B.	0.28	4.47	4.36	4.45	4.44
F. P.	0.28	4.28	4.57	4.27	
R. T.	0.11	2.27	2.26	2.37	2.26
		2.30*	2.25*	2.30*	2.25*

* Glycogen employed.

It is believed that 10 mg. of starch furnish a sufficient substrate for all ordinary conditions with human blood (except possibly in very severe diabetes), since practically identical results have been obtained with amounts of starch up to 40 mg., as shown in Table I. Glycogen offers no advantage over soluble starch, since the results are essentially identical. This is evident from the data given in Table II. The possible error of glycolysis would appear to be an entirely negligible one during a 15 minute period of incubation.

[18] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

The scheme we have followed in recording our results is an arbitrary one, but the figures obtained, ranging from 15 to 75, are satisfactory for expressing the variations in the diastatic activity of human blood. If it is desired to express the data in mg. of reducing sugar formed per 100 cc. of blood, this may readily be done by multiplying the above figures by 5. Some such method of recording the results would, obviously, be necessary in comparing the activity of the blood with that of other body tissues. It is believed that the salt content already present in the blood is sufficient to exercise a satisfactory activating influence on the diastatic enzyme.[19]

TABLE II.

The Comparative Diastatic Activity on Soluble Starch and Glycogen.

Case.	Blood sugar.	Soluble starch 10 mg.	Glycogen 10 mg.
		Reducing sugar formed.	
	per cent	*mg.*	*mg.*
F. P.	0.55	7.39	7.40
H. I.	0.17	4.23	4.12
F. M.	0.16	4.35	4.40
W. H.	0.16	3.37	3.24
A. K.	0.16	1.73	1.72
R. T.	0.11	2.27	2.30

DISCUSSION OF DATA.

The data which have been collected on the diastatic activity of human blood[20] have been arranged in tabular form under four general headings: normals, diabetics, nephritics, and miscellane-

[19] For discussion of factors which need to be borne in mind in estimating diastatic power, see Sherman, H. C., Kendall, E. C., and Clark, E. D., *J. Am. Chem. Soc.*, 1910, xxxii, 1073, and Kendall, E. C., and Sherman, H. C., *ibid.*, 1910, xxxii, 1087.

[20] We are indebted to Dr. Edward Quintard, Director, and to Drs. A. F. Chace and Ludwig Kast, and other members of the staff of the Department of Medicine for placing these cases at our disposal, and to Dr. R. C. Hood of the Laboratory Interne Staff, for valuable assistance. Our thanks are likewise due to members of the Laboratory Interne Staff for serving as our controls.

ous conditions. The results of other blood analyses, *viz.*, the sugar and CO_2-combining power and the non-protein nitrogenous constituents, have likewise been included for the reason that they furnish comparative data, and further, constitute an excellent index of the condition of the different cases.

The findings on six normal men are given in Table III. It will be noted that the activities in these subjects fall within narrow limits, 15 to 17. From the data that we have obtained, it would seem reasonable to conclude that normally the diastatic activity of the blood should not rise above 25, and an even safer limit might be between 15 and 20. With regard to the other analytical data, it will be noted that the uric acid for the last case appears

TABLE III.

The Diastatic Activity of the Blood in Normal Subjects.

Subject.	Age.	Sex.	Diastatic activity.	Blood sugar.	Uric acid.	Urea N.	Creatinine.
					Per 100 cc.		
	yrs.			*per cent*	*mg.*	*mg.*	*mg.*
C. E. W.	25	♂	17	0.12	3.9	13.2	1.4
W. O. H.	24	♂	17	0.11	3.0	13.6	2.3
J. A. K.	25	♂	17	0.10	3.3	14.8	1.1
A. L. L.	27	♂	17	0.10	4.3	14.1	1.1
R. C. H.	28	♂	16	0.10	3.3	14.1	1.2
W. M. H.	29	♂	15	0.11	4.8	12.8	1.0

high, and it may be said, concerning this subject, that he had recently recovered from a mild attack of acute nephritis.

By reason of the marked contrast to the normal and their importance, the observations on the diastatic activity of the blood in diabetes will next be considered. Data on thirteen cases have been collected in Table IV. Determinations of the non-protein nitrogenous constituents were made in many of these cases, but as the figures were essentially normal, they have not been included. It will at once be noted that the diastatic activity is uniformly from two to four times the figure normally observed —figures from 39 to 74. It will further be observed that, with the exception of Case 4, there is a rather close relationship between the blood sugar and the diastatic activity. Case 4 was

on a rigidly restricted diet throughout the period of observation and this would serve to explain the comparatively low figures for blood sugar with the high diastase figures, while, on the other hand, the high diastatic activity affords an explanation as to why the restriction in the diet was unable to bring the blood sugar down to normal. It should, perhaps, be noted that on October

TABLE IV.

The Diastatic Activity of the Blood in Diabetes.

Case.	Age.	Sex.	Date.	Diastatic activity.	Blood sugar.	CO_2-combining power (Van Slyke).
	yrs.		*1916–17*		*per cent*	
1. F. P.	51	♂	Nov. 17	74	0.55	54
			" 24	47	0.28	
2. S. I.	52	♂	Dec. 6	64	0.40	
3. L. O.		♀	Nov. 28	59	0.52	58
			" 10	59	0.17	49
			" 14	44	0.16	56
4. F. M.	26	♂	" 17	43	0.17	
			" 21	39	0.18	
			" 24	41	0.20	
5. G. A.	53	♂	" 26	57	0.44	
6. J. B.	65	♂	Dec. 23	56	0.30	
7. R. G.	57	♀	Nov. 1	53	0.43	56
8. I. A.	56	♀	" 1	53	0.26	60
9. F. A.	51	♀	Oct. 28	51	0.34	
			" 10	49	0.22	50
10. N. L.	55	♀	" 27	47	0.50	
			Dec. 6	46	0.28	
11. F. B.	47	♂	Nov. 29	45	0.28	56
12. T. H.	71	♂	Jan. 3	42	0.23	
13. L. M.	62	♀	" 3	42	0.26	
			" 9	24	0.12	

22 the blood sugar in this case was 0.29 per cent. A diastase estimation was, unfortunately, not made at that time. Case 13 cleared up quickly after admission to the hospital, and it will be observed that the fall in the diastatic activity corresponds with the fall in blood sugar. Higher blood sugars in one case than another, with the same diastatic activity, would appear to depend

largely upon the supply of carbohydrate (glycogen) available. The figures for the CO_2-combining power (Van Slyke), no one of which is much below normal, are apparently without special significance in this series.

A recent observation bearing on the influence of the administration of alkali may be of interest in this connection. The diastatic activity of the blood in a severe case of diabetes (J. S.), who was taking large quantities of sodium bicarbonate, was 26 and the blood sugar 0.44 per cent. Upon discontinuing the alkali therapy for 40 hours the blood diastase rose to 71 and the blood sugar to 0.58 per cent. The patient was again put upon alkali, but died several days later, before we were able to secure another specimen of blood. Although the question of the action of the alkali is important, the data we have so far been able to collect do not warrant a discussion of this subject. We have, however, made a few observations upon the action of alkali, and also of ether anesthesia, upon the blood diastase and sugar.

The diastatic activity of the blood has also been determined in two cases of carcinoma of the pancreas. In one (T. L.) a blood diastase of 45 and a blood sugar of 0.15 per cent was found, and in the other (D. M.) a diastase of 45 and a blood sugar of 0.14 per cent.

That diabetes is not the only condition in which an increase in the diastatic activity of the blood may be found, is shown by the data on cases of nephritis given in Table V, more than half of which gave abnormally high activities. In Case 1 the figure was 52 just before death. It will be noted that the cases with the increased diastatic activity almost invariably showed a hyperglycemia, and it would seem that the diastase stood in a causal relation to this. Attention was called, a year ago, to the high blood sugars frequently encountered in nephritis,[18] but at that time we were unable to offer a satisfactory explanation for the hyperglycemia. Judging from a comparison of the data on the diastatic activity and the urea, it would appear that nitrogen retention was generally accompanied by an increase in the blood diastase, probably due, likewise, to an inefficiency on the part of the kidney. In Cases 1, 3, and 11, in which a series of analyses was made, it will be noted that there is a striking parallelism

TABLE V.

The Diastatic Activity of the Blood in Nephritis.

Case.	Age.	Sex.	Diastatic activity.	Blood sugar.	Uric acid.	Urea N.	Creatinine.
					Per 100 cc.		
	yrs.			*per cent*	*mg.*	*mg.*	*mg.*
1. M. P.	25	♂	38	0.17	8.0	85	8.1
			41	0.15		123	11.6
			44	0.27		141	14.4
			52	0.22		156	12.1
2. E. P.	34	♀	42	0.21	11.0	85	17.6
3. J. B.	34	♂	32	0.14		60	7.5
			32	0.14	4.4	62	7.3
			41	0.16	6.0	135	9.7
			38	0.16	6.3	110	12.5
4. W. McD.	64	♂	35	0.16	6.3	30	2.7
5. M. B.	60	♀	34	0.16		85	3.2
6. C. K.	20	♀	34	0.18	2.3	16	2.1
7. D. S.	64	♂	33	0.20		86	4.1
8. J. G.	30	♀	31	0.13		22	5.0
9. N. W.	17	♀	30	0.32		72	6.1
10. J. v. M.	72	♂	30	0.14	4.5	20	1.8
11. J. M.	41	♂	21	0.13		28	2.9
			22	0.13	7.3	28	3.5
			25	0.11		39	1.9
			30	0.16	6.4	47	3.6
12. F. K.	42	♀	29	0.18	2.3	16	2.1
13. A. N.	54	♂	28	0.14		32	2.3
14. T. N.	43	♂	27	0.13	5.5	23	2.2
15. K. W.	42	♀	27	0.12	4.4	14	1.9
16. A. P.	40	♂	26	0.12		35	3.4
17. R. M.	29	♀	24	0.12		16	2.0
18. F. K.	43	♀	23	0.14		20	2.8
19. R. A.	30	♀	22	0.13	8.3	68	18.7
20. B. B.	23	♂	22	0.12		17	1.9
21. M. Y.	64	♂	21	0.14	5.5	29	1.5
22. J. G.	55	♂	20	0.14		16	2.5
			17	0.12	8.1	20	2.3
23. G. B.	38	♀	13	0.13	7.5	28	1.6

Case 1, CO_2-combining power = (2) 31; (3) 56, after alkali; (4) 44; died.
" 2, " " = 32, died.
" 3, " " = (2) 32.
" 9, " " = 20.
" 11, " " = (3) 47.
" 19, analyses after decapsulation of kidneys.

between the urea and the diastase. Case 9, with the highest blood sugar, showed the lowest CO_2-combining power.

Of the thirty-four cases included in Table VI under miscellaneous conditions, the first eight may be regarded as showing an increased diastatic activity. With the exception of Case 7, they all exhibited a slight hyperglycemia. It is interesting to note that when inquiry was made of Cases 3 and 7, both of whom were physicians and whose blood had been examined for reasons other than the blood sugar, a history of a previous transient mild glycosuria was obtained. Cases 1, 2, and 4 show a decided hyperglycemia, but the meager histories on these cases give no evidence of a glycosuria. Of the remaining twenty-five cases with diastase figures below 25, only three, Cases 16, 24, and 32, exhibit a decided hyperglycemia.

It would hardly seem profitable, in the present communication, to enter into an extended discussion of the rôle of diastases in metabolism. It should, perhaps, be noted here that the term diastase has been used in preference to amylase or glycogenase, for the reason that diastase is a broader term and might be regarded as covering action on both starch and glycogen.

Some text-books in mentioning the diastase of the blood state that the amount present is too small to be of any practical importance. It may be said, in this regard, that the 6 liters of blood of a normal subject of 70 kilos could convert 1 kilo of starch to sugar in 24 hours, if the activity of the blood remained at 20, according to the above method, during that time.

The character of the blood sugar curve, after the administration of glucose, has been the subject of a number of investigations. The blood diastase may possibly be the important determining factor here. The increased diastatic activity may likewise explain the inability to secure a storage of glycogen in the liver of diabetic animals.[21] It would seem logical to expect that an alimentary glycosuria should occur in individuals with an increased diastatic activity. Furthermore, the diastatic activity of the blood may, indeed, furnish a method of detecting cases of incipient diabetes.

Whether or not the diastatic activity of the blood is normally regulated by some internal secretion, derived, possibly, from the

[21] See Kramer, B., and Marker, J., *J. Biol. Chem.*, 1916, xxiv, p. xxiv.

TABLE VI.

The Diastatic Activity of the Blood in Miscellaneous Conditions.

Case.	Age.	Sex.	Diagnosis and remarks.	Diastatic activity.	Blood sugar.	Uric acid.	Urea N.	Creatinine.
						Per 100 cc.		
	yrs.				*per cent*	*mg.*	*mg.*	*mg.*
1. S. S.	32	♂	Bronchitis, died.	39	0.19		16	2.0
2. W. H.	47	♂	Lues.	34	0.16		13	2.3
3. E. H.	36	♂	Slight albuminuria and cylinduria, glycosuria noted on one occasion.	34	0.16	6.1	13	1.3
				29	0.11	5.1	12	1.7
4. A. M.	42	♀	Syphilis.	30	0.16		18	1.3
5. M. J.	43	♀	Fibrillation.	30	0.14		12	1.1
6. D. D.	38	♂	Gastric ulcer and gall-stones.	29	0.14		15	2.1
7. Z. D.	53	♂	Transient glycosuria noted on two occasions.	29	0.12	6.6	13	1.8
8. A. D.	35	♀	Visceroptosis.	28	0.14		19	1.5
9. O. E.	27	♂	Right side hemiplegia.	25	0.12		25	2.4
10. I. O'L.	36	♂	Sciatica.	24	0.12		20	2.0
11. S. S.	28	♂	Incipient tuberculosis.	23	0.13		14	1.7
12. R. T.	29	♂		23	0.11	5.8	14	1.3
13. J. R.	37	♂		23	0.13	6.2	20	1.2
14. D. A.	18	♂	Syphilis.	23	0.12	4.3	14	1.8
15. J. S.	53	♂	Carcinoma of stomach.	21	0.13		21	1.5
16. E. S.	45	♀	Infectious arthritis.	21	0.17		15	2.1
17. L. H.	32	♂	Tabes.	21	0.12		21	2.1
18. P. S.	50	♂	Duodenal ulcer.	21	0.12		19	1.4
19. R. M.	39	♀	Enteroptosis.	20	0.10		13	1.5
20. M. O.	27	♂	Chronic constipation.	20	0.12	5.0	17	2.6
21. B. P.	18	♀	Cystic ovaritis.	19	0.12		11	1.9
22. S. L.	37	♂	Neurasthenia.	18	0.12	3.2	12	1.4
23. M. S.	35	♂	Duodenal ulcer.	17	0.12		22	2.0
24. E. J.	49	♂	Cardiac with pleural effusion.	17	0.16		27	1.8
25. V. R.	37	♀	Lues, enteroptosis, and pyloric stenosis.	17	0.12		22	1.9
26. H. L.	64	♂	Hypertrophied prostate, pneumonia, died.	16	0.14	5.8	21	3.5
27. M. H.	57	♀	Raynaud's disease.	16	0.13	1.4	14	1.3
28. L. B.	55	♀	Lues.	16	0.11		11	1.1
29. D. S.	47	♂	Hypertension.	16	0.12	6.1	20	1.7
30. M. M.	55	♂	Hypertrophied prostate.	15	0.12	4.5	13	2.4
31. A. K.	28	♂	Pericarditis and arthritis.	15	0.13		17	2.3
32. B. C.	35	♀	Luetic cirrhosis.	15	0.16		12	1.6
33. H. D.	72	♂	Hypertrophied prostate, carcinoma of sigmoid.	15	0.12	6.1	22	1.9
34. J. B.	24	♀	Enteroptosis.	14	0.14		18	1.4

pancreas, it is impossible to say. For a discussion of the internal secretions in this connection, reference may be made to Macleod.[22]

Our studies on diastatic activity with this method were originally begun upon the ptyalin of the saliva, and it is planned to present a report of this work shortly. Other studies are in progress.

SUMMARY.

A simple method for the determination of the diastatic activity of human blood is described.

The diastatic activity of the blood in six normal human subjects ranged from 15 to 17 with this method. From the study of a considerable number of miscellaneous hospital cases, it would appear that the upper normal limit was 25. In a series of 13 cases of diabetes, the figures for the diastatic activity of the blood varied from 39 to 74. In a series of 23 cases of nephritis, 11 showed diastase figures ranging from 30 to 52. Of the 11 cases, 7 exhibited marked nitrogen retention.

The results suggest that the increased diastatic activity in both diabetes and nephritis (as shown by the analyses of the blood) may be the important factor in the production of the hyperglycemia in these conditions.

The increased diastatic activity of the blood in nephritis finds probable explanation in the decreased excretion of diastase in the urine, now well known in this condition, but it is not possible, at present, to offer a satisfactory explanation of the increased diastatic activity of the blood in diabetes.

A fall in the blood diastase would appear to afford a more reliable guide to the efficacy of the dietetic treatment in diabetes than either the blood sugar or urine sugar. Furthermore, an increase in blood diastase may constitute a very early sign of impending diabetes.

[22] Macleod, J. J. R., Diabetes: Its Pathological Physiology, London, 1913, 89.

THE AMINO-ACID NITROGEN CONTENT OF THE BLOOD OF VARIOUS SPECIES.

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The data thus far available upon the quantitative occurrence of amino-acid nitrogen in the blood of different species are far from complete. Furthermore, many of the figures heretofore reported have been obtained after preliminary removal of the blood proteins with alcohol, a procedure which has been shown to be undesirable from the standpoint of accuracy.[1] It was, therefore, believed that it would be of interest to make a fairly complete survey of the question of the quantity of amino-acid nitrogen in various bloods, making use of one method throughout, which had been found to be the most accurate available. The results thus obtained form the subject of the present paper.

Blood from various animals, as well as normal and pathological human blood, was obtained and the amino-acid nitrogen determined by the following method.[2]

Introduce into a flask approximately 0.4 gm. of ground (20 mesh) soy bean, 3 to 5 cc. of water, and 1 cc. of 3 per cent solution of crystallized NaH_2PO_4. Let stand a few minutes with occasional shaking. Run in 20 to 50 cc. of blood and allow to stand at room temperature for 30 minutes. Heat 0.01 N acetic acid to boiling, using five volumes of acid for one of blood. Run in the blood slowly and boil, stirring for ½ minute. Add the same amount of boiling water and boil with stirring for 1 minute. Filter hot through a folded filter and wash the casserole three times with 30 cc. portions of water, heating the water in the casserole and using a rubber-tipped stirring rod. Boil down the filtrate rapidly over a free flame in a casserole. Transfer to a small graduated flask or cylinder, choosing the size so as to obtain nearly the original volume of the blood. Wash the casserole three times with the smallest possible amount of water. The

[1] Bock, J. C., *J. Biol. Chem.*, 1916-17, xxviii, 357.

[2] This method has been previously described in detail by the writer.[1] It is repeated here for the sake of completeness.

volume after washing should not be more than about three-fourths of the final volume. Add enough trichloroacetic acid to make an approximately 3 per cent solution (either the solid acid or a corresponding amount of 50 per cent solution is used). Dilute to volume, shake, and let stand 30 minutes. Add 2 gm. of kaolin, shake well, centrifuge, and filter. Transfer an aliquot part (15 to 40 cc.) of the filtrate to a small flask (50 to 100 cc.), add two or three beads, one drop of alizarin indicator, bring to a boil, and keep slowly boiling (simmering) until the indicator turns. Add 1 to 2 cc. of N potassium hydroxide, and boil 1 to 2 minutes to remove the ammonia. Make acid with a few drops of acetic acid and evaporate to a small volume. The whole amount may be used in the micro Van Slyke apparatus or the liquid transferred to a small graduated test-tube, diluted to a definite volume, and aliquot portions measured out by means of the burette of the Van Slyke apparatus.[3]

The heat coagulation gives clear and easily filterable precipitates with freshly drawn blood; *i.e.*, blood not over 24 hours old. If the blood has been drawn for more than that time, it often happens that the filtrate from this precipitation is not entirely clear and the solution filters very slowly. The same thing has been found with laked or frozen blood. In such cases the blood must be precipitated with nine volumes of 2.5 per cent trichloroacetic acid and treated as described in a previous paper.[1]

The blood of larger animals, such as the ox, calf, sheep, and pig, was obtained from slaughter houses and immediately (1 to 2 hours after slaughtering) used for analysis. The blood of cats and dogs was obtained from the carotid artery. The blood was drawn about 12 hours after the last feeding. Normal human blood was drawn from a vein in the forearm (usually the median basilic).[4] The normal and most of the pathological bloods were drawn 3 to 4 hours after breakfast. The blood of birds was obtained by cutting the throat and letting the blood run into a bottle containing potassium oxalate. 40 to 50 cc. of animal and normal human blood were used for the analysis. The amounts of pathological bloods obtained from the wards varied greatly, but with few exceptions there were always more than 15 cc. used for analysis. These bloods were delivered in small bottles and in order to prevent waste, the blood was weighed out instead of measured, pouring it into the previously tared flask containing the soy bean mixture. The weight in gm. was divided by 1.06, the mean specific gravity of human blood. For the sake of comparison the results are tabulated at the end of the paper.

Table I shows the amino-acid nitrogen content of the blood of various mammals, including the ox, sheep, pig, cat, and dog.

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121; 1915, xxiii, 407.

[4] The pathological blood was furnished through the courtesy of Dr. A. O. Gettler and other members of the staff of Bellevue Hospital, to whom the writer expresses his thanks.

The figures for each species are constant. Van Slyke and Meyer,[5] using the alcohol precipitation method, find from 3 to 5 mg. of amino-acid nitrogen per 100 cc. of blood of dogs which had been fasting from 20 to 24 hours. Costantino[6] reports findings of 10 mg. of amino-acid nitrogen in 100 gm. of blood obtained from dogs during full digestion. His analysis of pig blood shows 10 mg. of amino-acid nitrogen per 100 gm. of blood. Costantino uses the Sörensen formol titration method after drying the blood at 70°C. and extracting with 10 per cent alcohol in the presence of barium salts. György and Zunz,[7] using the alcohol precipitation method (removing urea by treatment with soy bean and subsequent aeration) find 4.8 mg. of amino-acid nitrogen in 100 cc. of blood obtained from dogs fasted for 24 hours. In the present work, dog blood was found to contain an average of 7.47 mg. of amino-acid nitrogen per 100 cc. of blood, and pig blood 8.43 mg. per 100 cc. of blood. These higher figures in the dog are probably due to the more complete extraction of the amino-acid nitrogen obtained in the present work. We cannot explain Costantino's high results for pig blood.

The amino-acid nitrogen of bird blood (Table II) is, roughly speaking, three times as high as that of mammals. The individual and gross variations are in proportion to those of mammalian blood. Costantino, using the formol method[6] finds 20 mg. of amino-acid nitrogen per 100 gm. of turkey blood, agreeing closely with the findings in the present paper.

The distribution of the amino-acid nitrogen between plasma and corpuscles in certain species was studied by Costantino[6] and by György and Zunz.[7] Costantino finds that serum and corpuscles during fasting are constant in their amino-acid nitrogen. His findings show 4.4 mg. of amino-acid nitrogen per 100 gm. of dog serum, and 100 gm. of the corpuscles of ox blood containing 3.2 mg. of amino-acid nitrogen. Turkey blood analyzed by Costantino showed 3 mg. of amino-acid nitrogen in 100 gm. of serum and 34 mg. in 100 gm. of corpuscles. György and Zunz find 1.7 mg. of amino-acid nitrogen in the plasma of 100 cc. of

[5] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.
[6] Costantino, A., *Biochem. Z.*, 1913, li, 91; 1913, lv, 403.
[7] György, P., and Zunz, E., *J. Biol. Chem.*, 1915, xxi, 511.

dog blood and 3.1 mg. in the corpuscles of 100 cc. of the same blood, the corpuscle content being calculated by difference.

In the present work the whole blood was first analyzed. Samples of equal volumes were centrifuged for ½ hour at high speed. The plasma which had separated out was withdrawn by means of a pipette, physiological salt solution added in its place, and the sample was mixed and centrifuged again. This procedure was repeated four times. After all the clear supernatant fluid had been withdrawn down to the light colored layer of leukocytes, the corpuscles were laked with water, transferred to a flask, and the liquid was diluted approximately to the same volume as the plasma plus the washings. As the laked blood gives trouble with the heat coagulation procedure the direct trichloroacetic acid precipitation was used after treatment with soy bean. Table III shows the results obtained from mammalian and bird blood. In mammalian blood the corpuscles show slightly higher figures than the plasma. The difference in bird blood is pronounced, the corpuscles containing about two-thirds of the total amount of amino-acid nitrogen. These findings agree in general with those of the authors mentioned above.

In view of the fact that the corpuscles contain a large proportion of the amino-acid nitrogen of the blood, they should be included in experimental or clinical work on the nitrogen content of the blood.

The normal human bloods are reported in Table IV. It will be noted that the figures are remarkably constant for different individuals. The average figure is 7.13 mg. with a maximum of 7.9 mg. and a minimum of 6.13 mg. per 100 cc. of blood, approximately those of other mammals. Sex appears to be without influence. The placental blood tends to be distinctly higher.

The pathological bloods (Table V) were taken at random from ward cases in Bellevue Hospital and thus serve to show the possible variations found in a wide variety of conditions. In these cases variations occur from 4.5 mg. to 30 mg. per 100 cc. of blood. The most pronounced variations from the normal were found in nephritis. Of three typhoid cases, two are decidedly below normal. Jaundice, cardiac cases, carbuncles, rheumatic fever, hyperthyroidism, and cirrhosis of the liver show an increase over

the normal. The remaining cases investigated give the same results as normal bloods.

The nephritic bloods are being studied further to find whether the amino-acid nitrogen tends to parallel the total non-protein nitrogen in the blood of these cases.

TABLE I.

Amino-Acid Nitrogen per 100 Cc. of Blood.

Sample No.	Source.	N*	Sample No.	Source.	N*
		mg.			*mg.*
4	Ox (oxalated).	6.17	42	Pig (defibrinated).	8.37
125	" "	6.22	43	" "	8.49
10	" "	6.43	Average.		**8.43**
5	" "	6.66			
125a	" (defibrinated).	6.67	14d	Cat "	8.12
9	" (oxalated).	6.89	14b	" "	8.13
3	" "	7.04	14c	" "	8.64
Average.		**6.58**	14a	" "	9.83
			Average.		**8.68**
100	Calf (defibrinated).	6.29			
8	" (oxalated).	6.66	97	Dog "	6.68
18	" (defibrinated).	6.81	52	" "	6.87
18a	" "	7.60	113	" "	6.87
Average.		**6.84**	51	" "	7.15
			114	" "	7.47
6	Sheep (oxalated).	6.84	116	" "	7.88
13	" defibrinated).	7.50	98	" "	8.37
12	" "	7.79	99	" "	8.48
11	" "	7.82	Average.		**7.47**
7	" (oxalated).	8.19			
Average.		**7.63**			

* The data are arranged in sequence for each group.

TABLE II

Amino-Acid Nitrogen per 100 Cc. of Blood (Oxalated).

Sample No.	Source.	N*
		mg.
26	Chicken.	17.81
36	"	19.84
23	"	20.64
24	"	21.32
25	"	21.58
110	"	21.93
107	"	23.78
Average.....		**20.99**
35	Duck.	20.22
32	"	20.88
34	"	20.95
33	"	21.55
27	"	22.98
Average.....		**21.32**
101	Turkey.	20.00
102	Goose.	16.97
104	"	17.16
105	"	18.49
103	"	19.20
106	"	21.18
Average.....		**18.60**

* The data are arranged in sequence for each group.

TABLE III.

Sample No.	Source.	Amino-acid nitrogen per 100 cc. of blood.		
		Whole blood.	Plasma.	Corpuscles.
		mg.	*mg.*	*mg.*
100	Calf.	5.69	3.06 3.13	3.59 3.48
100a	"	6.30	3.33 3.33	3.84 3.28
106	Goose.	21.18	6.60 6.07	14.78 15.95
107	Chicken.	23.78	6.63 7.97	14.20 16.00
110	"	21.93	5.31	19.12
125	Ox.	6.22 6.77	3.05 2.99	4.70 4.87

TABLE IV.

Amino-Acid Nitrogen per 100 Cc. of Human Blood.

Sample No.	Sex.	N*
	Normal venous blood.	
		mg.
47	♂	6.13
15	♂	6.40
57	♂	6.58
60	♂	6.75
58	♂	6.78
77	♂	7.00
83	♂	7.01
86	♂	7.11
21	♂	7.18
30	♂	7.27
55	♀	7.29
48	♂	7.31
16	♂	7.38
22	♂	7.38
19	♂	7.58
65	♂	7.66
56	♀	7.67
46	♀	7.90
Average.....		**7.13**
	Placental blood.	
20		6.78
38		7.51
28		8.90
31		9.76
138		11.80
139		12.15
Average.....		**9.48**

* The data are arranged in sequence for each group.

TABLE V.

Amino-Acid Nitrogen per 100 Cc. of Pathological Human Blood.

Sample No.	N.	Diagnosis.	Sample No.	N.	Diagnosis.
	mg.			*mg.*	
39	7.78	Syphilis.	85	8.07	Nephritis.
40	7.98	"	87	6.97	Cardionephritis.
44	6.54	Ulcer on penis.	88	8.27	Rheumatic fever.
45	8.34	No symptoms.	89	7.01	Cardiac.
49	8.49	Nephritis.	90	9.38	Nephritis.
50	8.38	"	91	5.72	Carcinoma of liver.
53	7.11	Cardionephritis.	92	7.50	Nephritis.
54	17.50	Nephritis, alcoholism.	93	6.52	"
59	6.77	No record.	94	8.02	"
61	7.27	Nephritis.	95	11.28	Parenchymic nephritis.
62	8.78	No record.	108	8.13	" "
63	8.31	Jaundice.	109	8.04	Nephritis.
64	7.25	Nephritis.	110	8.49	"
66	8.62	Cardiac.	111	7.55	Parenchymic nephritis.
67	7.80	Diabetes.	112	8.69	Cardionephritis.
68	7.86	Cardionephritis.	115	8.87	"
69	6.47	Chronic nephritis.	117	6.66	Nephritis.
70	6.93	Typhoid.	118	8.24	Parenchymic nephritis.
71	8.70	Gout, rheumatism, nephritis.	120	10.98	Nephritis.
			121	9.82	"
72	5.88	Typhoid.	122	11.17	Colic, uremia.
73	5.45	"	123	8.41	Cardionephritis.
74	4.45	Nephritis.	124	29.98	Uremia.
75	15.10	"	126	9.78	"
76	7.52	Arteriosclerosis.	127	7.25	Nephritis.
78	9.05	Nephritis.	128	7.45	Delirium tremens.
79	5.90	Uremia.	129	9.91	Nephritis.
80	6.05	Nephritis.	130	10.45	Cirrhosis of liver and cardiovalvular case.
81	6.65	"			
82	8.20	Carbuncles.	131	9.58	Hyperthyroidism.
17	6.89	Nephritis.	132	6.46	Parturition.
84	6.24	"	133	6.63	Diabetes.

THE SEPARATION AND ESTIMATION OF BUTYRIC ACID IN BIOLOGICAL PRODUCTS. I.*

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Duclaux[1] has determined approximately quantitatively the individual volatile organic acids of the series from formic to caproic acids, inclusive, by estimating the acidity of the separate distillates. The individual acids are found to distil at a fixed rate, uninfluenced by the presence or absence of other volatile substances. The acids are determined, consequently, by their rate of distillation as a physical constant. This method was used by Jensen[2] to determine the volatile aliphatic acids in cheese, and by Dox and Neidig[3] to determine the volatile aliphatic acids in corn silage. Luck[4] separated formic, acetic, propionic, and butyric acids by the different solubilities of their barium salts in absolute alcohol. Haberland[5] could not duplicate Luck's findings but separated approximately quantitatively acetic and butyric acids by the difference in solubilities in water of their silver salts. The more or less complete solubility of these salts either in the alcohol or in the water renders impossible a quantitative separation of these acids in the manner described in the

* Read at the Fifty-third Meeting of the American Chemical Society in New York, September 25–30, 1916. Published by the permission of the Secretary of Agriculture.

[1] Duclaux, E., *Ann. sc. l'école normale supérieure*, 1865, ii, 270; Traité de microbiologie, Paris, 1900, iii, 384; *Ann. chim. et phys.*, 1874, series 5, ii, 289; 1886, series 6, viii, 542.

[2] Jensen, O., *Landwirtsch. Jahrb. Schw.*, 1904, xviii, 319.

[3] Dox, A. W., and Neidig, R. E., *Iowa Agric. Exp. Station Research Bull. 7*, 1912.

[4] Luck, E., *Z. anal. Chem.*, 1871, x, 184.

[5] Haberland, K. R., *Z. anal. Chem.*, 1899, xxxviii, 217.

literature. In a previous paper[6] from this laboratory it has been shown that lactic acid may be separated quantitatively from formic, acetic, and citric acids by the fractional distillation of their ethyl esters, and from propionic and butyric acids by the different solubilities of their quinine salts in carbon tetrachloride. The separated lactic acid was estimated as quinine lactate and identified by its melting point. The present paper describes a method for the separation of butyric acid from formic and acetic acids by treating their barium salts with an excess of quinine sulfate, dissolving the quinine butyrate in carbon tetrachloride, and weighing as quinine butyrate, which is identified by its melting point.

For the work recorded here the chemically pure carbon tetrachloride of commerce, found free from residue by volatilization of 50 cc., was used. The pure ethyl acetate of commerce was washed with water and dried over calcium chloride before distilling. The portion distilling between 77° and 77.5° was used. Petroleum ether was redistilled and the portion boiling between 55° and 75° reserved for use.

The purest formic and acetic acids of commerce were diluted suitably with distilled water, and the strength of the solution in each case determined by titration with a solution of barium hydroxide of known strength, approximately 0.1 N, with phenolphthalein as an indicator.

Pure butyric acid was prepared from ethyl butyrate, boiling at 120.9–121.1° (corrected) and under 763 mm. pressure obtained by repeated fractionation of commercially pure ethyl butyrate. This was hydrolyzed by heating with an excess of barium hydroxide solution under a return condenser. The excess of barium hydroxide was converted to the carbonate by passing carbon dioxide through the solution, the barium carbonate was removed by filtration, and by boiling the filtrate was freed from carbon dioxide. Dilute sulfuric acid (1:3) was added in excess, the precipitate of barium sulfate was filtered, and the filtrate was distilled under diminished pressure. The distillate containing the butyric acid was diluted to definite volume, and the strength of the solution was determined by titration with a solution of

[6] Phelps, I. K., and Palmer, H. E., *J. Am. Chem. Soc.*, 1917, xxxix, 136.

barium hydroxide of known strength, approximately 0.1 N, with phenolphthalein as an indicator.

Pure propionic acid was prepared from ethyl propionate, boiling at 98.7–99.0° (corrected) at 760 mm. The ethyl propionate was hydrolyzed by heating under a return condenser with an excess of barium hydroxide solution, and a solution of propionic acid was obtained from this in a manner similar to that described above for butyric acid. The strength of the solution of propionic acid was estimated by titration in the presence of phenolphthalein as an indicator, with a solution of barium hydroxide of known strength, approximately 0.1 N.

Pure quinine sulfate and pure quinine were prepared as described in the paper by Phelps and Palmer,[6] to which reference has been made. The quinine salts of formic, acetic, propionic, and butyric acids were prepared by adding the calculated molecular quantities of the pure quinine dissolved in 95 per cent alcohol to the separate acids and evaporating the solution to dryness under diminished pressure at 15 mm. The quinine butyrate was recrystallized from ethyl acetate solution by diluting to twenty times its volume with petroleum ether, when, on standing for a half hour without further stirring, crystals formed. These were gathered on paper and dried in a vacuum desiccator over potassium hydroxide. If the solution was stirred immediately after adding the petroleum ether, the quinine butyrate collected as a sticky mass on the sides of the beaker and was converted to the crystalline form more slowly. The quinine butyrate so obtained had a melting point of 77.5° (uncorrected). The approximate solubilities of the quinine salts of the acids in carbon tetrachloride, together with their melting points, are given in Table I. The solubilities were determined approximately by allowing each substance to stand in contact with a definite volume of carbon tetrachloride in a stoppered flask for 72 hours, filtering, and weighing the residue left on evaporation of the carbon tetrachloride after drying in a water-jacketed vacuum oven at 50°. It is seen from Table I that a separation of quinine butyrate from quinine formate and quinine acetate can be made by the difference in their solubilities in carbon tetrachloride, but the difference in solubility of quinine propionate and quinine butyrate is not sufficient for a complete separation.

TABLE I.

Melting Points and Approximate Solubilities of Quinine Salts.

	Melting point (uncorrected).	Approximate solubility in carbon tetrachloride.
	°C.	
Quinine formate	110.0–113.0	1 in 16,000
" acetate	124.0–126.0	1 " 2,000
" propionate	110.5–111.0	1 " 450
" butyrate	77.5	1 " 25
" sulfate	214.0	1 " 40,000

Definite portions of the solution containing the butyric acid, either alone or in the presence of other acids, were titrated with a solution of barium hydroxide of known strength, approximately 0.1 N, in the presence of phenolphthalein. To the neutral solution was added a slight excess of a solution of neutral quinine sulfate in hot water, the amount of quinine sulfate necessary to precipitate the barium completely as barium sulfate being calculated from the amount of barium hydroxide required. The solution was cooled by standing in ice water and filtered through asbestos as soon as the precipitate of barium sulfate had subsided. The residue was rinsed three times with portions of 10 cc. of distilled water. The filtrate, containing the quinine salts of any of the volatile organic acids that may be present, was evaporated to dryness by distillation under diminished pressure, preferably as low as 15 mm., in order to minimize the formation of quinotoxine from the quinine. The residue was dissolved from the sides of the flask with 10 cc. of alcohol, the alcohol removed by distillation under diminished pressure, and this residue dried by allowing a gentle current of air to pass through the flask. The dry residue, which was generally amorphous, was allowed to stand for 18 hours with 20 cc. of carbon tetrachloride, or with an amount sufficient to dissolve the quinine butyrate which might be present, as shown by the solubility given in Table I. If the original residue was amorphous, supersaturated solutions tended to form and some of the quinine salts of the other acids remained in solution with the quinine butyrate, necessitating retreatment with carbon tetrachloride to separate them. The solution was filtered, and the residue washed on the filter with carbon tetrachloride. The carbon tetrachloride solution, contained in a 100 cc.

Erlenmeyer flask, was evaporated to dryness at room temperature by allowing a rapid current of air to pass over the solution by means of a glass tube suspended just above the surface of the solution. If the residue was amorphous it was dissolved in ethyl acetate and after evaporation at room temperature by a rapid current of air the quinine butyrate was obtained in crystalline condition. To the residue, dried in a vacuum desiccator over potassium hydroxide, 20 cc. of carbon tetrachloride were added, and allowed to stand in the stoppered flask 18 hours to dissolve the quinine butyrate. The solution was filtered and the residue washed with carbon tetrachloride. The undissolved residue was allowed to stand in a stoppered flask for 18 hours with 10 cc. of carbon tetrachloride to dissolve any quinine butyrate which remained, and the solution was then filtered. The filtrates were evaporated at room temperature by allowing a rapid current of air to pass over the solution. When the quinine butyrate obtained in this manner from carbon tetrachloride by evaporation was amorphous, it was dissolved in a small amount of ethyl acetate, from which solution, after evaporation at room temperature by allowing a rapid current of air to pass over the solution, it was obtained in a crystalline form which could be dried in a vacuum desiccator over potassium hydroxide. The dried residue was weighed as quinine butyrate and identified by its melting point. If the melting point showed the presence of impurities, the residue was purified by another treatment with carbon tetrachloride. When it failed to appear crystalline after this purification the quinine butyrate was recrystallized as given above for the purification of the quinine butyrate when prepared for the experimental work. The crystals which formed on standing a few minutes were filtered, dried in the air, and identified by their melting point.

The results given in Table II show that, if the precautions are taken to avoid decomposition of the quinine butyrate by evaporating the solutions at low temperature and drying in a vacuum desiccator over potassium hydroxide, butyric acid may be estimated by weighing as quinine butyrate when present alone or when associated with formic acid and acetic acid, and may be identified as such by its melting point. When propionic acid is present, as in Experiments 11, 12, and 13, it is seen that some of the quinine propionate is dissolved with the quinine butyrate

in the carbon tetrachloride, so that a partial separation only in this way is possible. If the residue from the distillation under diminished pressure was in crystalline form, as was the case in Experiment 3 of Table II, it was found that the tendency of the quinine acetate to form a supersaturated solution in the carbon tetrachloride was so small that a further treatment of the residue from the evaporation of the carbon tetrachloride solution for the separation of these salts was unnecessary. The residue left undissolved by the carbon tetrachloride, however, was allowed to stand 18 hours with another 10 cc. of carbon tetrachloride, in order to dissolve the quinine butyrate completely. This solution was added to the solution obtained by the first extraction, before evaporating to obtain the residue, the purity of which after weighing was found by its melting point.

TABLE II.

Estimation of Butyric Acid after Separation from Formic, Acetic, and Propionic Acids.

No.	Acids taken.				Quinine butyrate found.	Butyric acid calculated.		Melting point of quinine butyrate.
	Formic.	Acetic.	Propionic.	Butyric.				
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*°C.*
1				0.1129	0.5271	0.1126	99.7	77.5–78.0
2				0.1382	0.6287	0.1343	97.2	
3		0.0972		0.0691	0.3119	0.0666	96.4	77.5–78.0
4		0.1037		0.1129	0.5310	0.1134	100.5	75.5
5		0.0519		0.1694	0.7395	0.1580	93.2	77.5–78.0
6	0.0804			0.0691	0.2993	0.0639	92.4	77.5–78.0
7	0.1119			0.1129	0.5189	0.1108	98.2	77.0
8	0.0560			0.1694	0.7112	0.1519	89.7	76.5–77.0
9	0.0560	0.0519		0.0565	0.2672	0.0571	101.0	76.0–76.5
10	0.1119	0.1037		0.0565	0.2507	0.0535	94.8	74.5–75.0
11			0.1414	0.1129	0.6042	0.1291	114.3	Softens at 76.0–77.0 Melts at 92.0–93.0
12			0.1414	0.0565	0.3722	0.0795	140.7	92.0–95.0
13			0.0707	0.1694	0.9962	0.2128	125.6	Softens at 75.0–76.5

SUMMARY.

The melting points and approximate solubilities of quinine propionate and quinine butyrate in carbon tetrachloride were determined. The solubilities of the propionate and butyrate

are so nearly the same that only a partial separation can be effected by the method described. The solubility of the propionate is so much greater than that of the formate that propionic and formic acids can be readily separated. It has been shown that butyric acid may be separated from mixtures containing formic, acetic, and butyric acids by means of the solubility of quinine butyrate and the insolubility of quinine formate and quinine acetate in carbon tetrachloride; further, the butyric acid may be estimated by weighing the quinine butyrate, which may be crystallized and identified by its melting point.

THIOBARBITURIC ACID AS A QUALITATIVE REAGENT FOR KETOHEXOSE.

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(Received for publication, January 20, 1917.)

According to Fenton and Gostling,[1] all hexoses are convertible into hydroxymethylfurfural. This substance is, however, much more easily formed from the ketoses than from the aldoses. It is upon this fact that the orcinol, resorcinol, and phloroglucinol tests depend. Fenton and Gostling obtained several times as much bromomethylfurfural from ketohexoses or ketohexose-yielding substances as they did from aldohexoses. By heating with 0.3 per cent oxalic acid under three atmospheres' pressure Van Eckenstein and Blanksma[2] obtained practically a 20 per cent yield of hydroxymethylfurfural from fructose, while from glucose they obtained but 1 per cent. The writer has been able to convert 70 to 75 per cent of fructose into hydroxymethylfurfural by heating with concentrated hydrochloric acid. Under the same conditions sucrose yielded 60 to 70 per cent of the theoretical amount of its fructose in the form of the furfural derivative. The method of determination consisted in precipitating the hydroxymethylfurfural with thiobarbituric acid[3] in the presence of 12 per cent hydrochloric acid, drying, and weighing the precipitate. The product had a reddish orange color and was appreciably soluble in water. Had solubility corrections been applied, the above percentages would no doubt have been higher. Analysis of the product showed a nitrogen content of 11.20 per cent, whereas the

[1] Fenton, H. J. H., and Gostling, M., *J. Chem. Soc.*, 1899, lxxv, 423; 1901, lxxix, 807.

[2] Van Eckenstein, W. A., and Blanksma, J. J., *Ber. chem. Ges.*, 1910, xliii, 2355.

[3] Dox, A. W., and Plaisance, G. P., *J. Am. Chem. Soc.*, 1916, xxxviii, 2156.

calculated value for the condensation product, hydroxymethylfurfuralmalonylthiourea is 11.11 per cent.

When glucose was subjected to the above treatment only a very slight precipitate resulted. Even after long continued heating with acid, 0.2 gm. of glucose gave only 0.001 to 0.002 gm. of precipitate. Lactose and maltose gave somewhat larger amounts of hydroxymethylfurfural, especially after prolonged heating. As suggested by other investigators, this indicates a gradual molecular rearrangement, possibly from aldose to ketose.

On the other hand, prolonged heating of fructose decreases the yield of hydroxymethylfurfural, the latter substance undergoing decomposition into levulinic acid. The yield is likewise slightly decreased by using 12 to 15 per cent hydrochloric acid instead of the concentrated acid. When acid of this strength is used, no precipitate whatever can be obtained from glucose or other aldohexoses.

The thiobarbituric acid test gave positive results when applied to the following carbohydrates, all of which contain a ketose: sucrose, raffinose, tagatose, *l*-sorbose, and inulin. The following gave negative tests: glucose, mannose, galactose, lactose, maltose, starch, cotton, filter paper, glucosamine, α-methylglucoside, arabinose, and xylose.

A noteworthy fact is that the pentoses are not as easily converted into furfural as are the ketohexoses into the corresponding hydroxymethyl derivative. Arabinose and xylose do not form furfural until the acid concentration is almost 20 per cent. However, the presence of furfural can easily be tested in the distillate by means of aniline acetate paper.

It is recommended that the thiobarbituric acid test for ketohexoses be applied as follows: The substance to be tested is placed in a test-tube and sufficient hydrochloric acid and water are added to bring the acid concentration to 12 per cent. The tube is heated over a free flame until boiling begins. It is then cooled under the tap and finally a few drops of a thiobarbituric acid solution (in 12 per cent HCl) are added. If a ketohexose was originally present, an orange-colored precipitate forms on standing. If only aldoses were present, the solution may become yellow but no precipitate results. Barbituric acid cannot be used in place of thiobarbituric, for the reason that the condensation product is much more soluble.

THE GROWTH OF ISOLATED PLANT EMBRYOS.

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Delayed germination or the resting period of seeds presents not only one of the most interesting problems in plant life but also one of the most difficult, owing mainly to the many conditions governing the normal germination of seeds. Space does not permit of even a brief historical sketch of the abundant investigations that have been made concerning this subject. Suffice it to say that these studies have brought forth explanatory theories ranging from zymogenic changes occurring in the embryos and cotyledons to the toughness and non-permeability of the integument to moisture and gases, either or both being more or less dependent on the physical conditions affecting the seed, among which might be named temperature, pressure, moisture, light, and the surrounding media, whether gaseous, liquid, or solid.

From one point of view it would seem that there is a rest period of a longer or shorter duration in the life of most seeds which is limited by internal changes, such as the accumulation of ferments or other chemical changes normally dependent on time, while from another point of view it would seem that there is no real resting period and that germination is delayed because of lack of favorable conditions or proper stimulant. This latter view is more or less proved by the many instances where investigators have greatly shortened the apparent resting period of seeds by physical and chemical methods and have increased the percentage germination of certain seeds by like methods.

Following this line of thought, it might be added that after the rest period is ended the embryo is dependent on the food supply stored in the cotyledons. The young plant consumes part of this food supply which is sufficient to cause it to grow until it

has generated organs capable of utilizing nutriment from external sources—then the cotyledons, being of no further value, shrivel up and finally drop off.

The embryos of different seeds are found to differ widely in size and nature, and the food which serves to nourish them during their early growth varies greatly in character and composition. In the case of the persimmon seed, for example, the embryo consists of two perfectly formed leaves attached to a well defined caulicle—this minute plant draws its early nourishment from cotyledons which, like the seed of the date, closely resemble vegetable ivory in appearance. Among the seeds containing highly specialized embryos might be named the okra, papaw, corn, bean, and many others.

Some years ago during the progress of certain experiments which involved the study of seeds, one of us (Kastle) observed these highly specialized embryos and became interested especially in the embryos of the okra and lima bean. The embryo of the lima bean consists of two perfectly formed leaves attached to a caulicle about 0.75 cm. in length. Finding that this embryo could be easily dissected out, it seemed of general interest to determine if it would grow when deprived of its normal food supply from the cotyledons, and ultimately to establish some of the relations existing between an embryo and the adjoining cotyledons. Other investigations being in progress, it was impossible to continue this research and it was only last spring that during the progress of investigations concerning the study of beans in this laboratory, an embryo of the lima bean was dissected out and planted in moist sand. At the expiration of 24 hours it was noticed that the caulicle had doubled in length but the leaves remained unchanged in size and color while the small bud between the colorless leaves developed a pale green color. There was no further development observed and at the end of 8 days the young plant had dried up and was dead, having developed no lateral roots.

Here was found ample evidence of life and it remained to be determined what substances would nourish the embryo and bring about the normal growth of the plant. With this idea in view a series of experiments was planned to determine the food requirements of isolated lima bean embryos, and to establish the rela-

tions existing between the cotyledons and the embryos during early growth.

Knudson[1] has published some results concerning the toxicity of galactose and the more or less stimulating effect of certain carbohydrates on the vetch, pea, and other seeds. In these experiments he has grown plants from the seeds that had been planted in nutrient solutions containing 1 per cent agar plus different carbohydrates. In his experiments these carbohydrates are supplemental to the food supply stored in the cotyledons, and he does not prove the necessity of any of these compounds for the growth of the young embryo.

In the literature at our command we have failed to find any reference concerning the early food requirements of a seed embryo except that the reserve material stored in the cotyledons, upon hydrolysis, furnishes the nourishment required by the embryo during early growth.

In these experiments perfect beans were selected, care being taken that the integuments were not injured. After carefully removing the integument, the embryo was dissected out under sterile conditions and dropped into sterile distilled water. The embryos were immediately transplanted into test-tubes that were half filled with the different nutrient media, all operations being carried out in a dust-proof closet. The medium in each case was composed of distilled water containing 0.65 per cent of well washed agar plus the different nutrients, as shown in the table.

It will be seen from the table that agar alone does not support the growth of the embryo of the lima bean. The embryo swells to about twice its original size, the leaves turn pale green in 3 days, and then etiolate, and the plant dies in 9 days, no lateral roots having been developed.

When glucose was added to the agar a good growth was obtained, the radicle above the agar and leaves turning green in 48 hours and the growth reaching 2 inches in 14 days, having developed a good root system.

Glucose plus Hopkins' plant food solution gave good growth, the size being greater than when glucose alone was used.

[1] Knudson, L., *Ann. Missouri Bot. Gardens*, 1915, ii, 659.

Lima Bean Embryos.

No.	Media.	Result.
1	Agar (0.65 per cent) in distilled water. Control	Very small growth. Dead in 9 days.
2	Glucose (2 per cent)	Good growth.
3	" (2 " ") + 1 cc. Hopkins'* plant food solution	Best "
4	1 cc. Hopkins' plant food solution	No "
5	Dry lima bean cotyledons, ground (2 per cent)	" "
6	Dry lima bean cotyledons, ground, + 1 cc. Hopkins' solution	" "
7	Dry lima bean cotyledons, ground, + 2 per per cent glucose	Good "
8	Sucrose (2 per cent)	" "
9	Lactose (2 " ")	Fair "
10	Raffinose (2 " ")	" "
11	Mannite (2 " ")	Very small growth. No lateral roots.
12	Maltose (2 " ")	Small growth.
13	Starch (2 " ")	No "
14	Sprouting green cotyledons (2 per cent) (reducing sugars present)	Small "
15	Sprouting green embryos (2 per cent) (reducing sugars present)	" "
16	Dry cotyledons (2 per cent) (reducing sugars absent)	No "

* Hopkins, C. G., and Pettit, J. H., Soil Fertility Laboratory Manual, Boston, 1910, p. 22.

Hopkins' solution, when used alone with agar gave no growth greater than the control. It has been shown here that a soluble reducing sugar is necessary for growth.

Dry lima bean cotyledons were pulverized and placed in boiling distilled water. This solution plus agar gave no growth.

This same solution of the dry cotyledons plus Hopkins' solution gave no growth, but good growth was obtained when 2 per cent glucose was added.

Sucrose plus agar gave good growth, practically the same as glucose.

2 per cent lactose and raffinose gave fair growth.

Mannite gave practically no growth, the leaves turning pale

green and the entire embryo possibly doubling its size in 3 weeks. No lateral roots were formed.

Maltose gave small growth, and soluble starch gave no growth.

Some beans were allowed to sprout in moist sand, the cotyledons turning green. The green cotyledons were macerated and a water extract was made of them. This extract when filtered reduced Fehling's solution, showing the presence of reducing sugars. When this extract was added to agar small growth was obtained.

Some beans were allowed to germinate and sprout for several days on moist cotton. The embryos increased greatly in size and turned green. A water extract of the green sprouting embryos was made which reduced Fehling's solution. An embryo when placed in an agar solution of this extract gave a small growth.

Dry cotyledons of the lima bean, when pulverized and placed in boiling water, do not reduce Fehling's solution and will not support the growth of its own embryo.

SUMMARY.

In these experiments attempts were made to nourish the embryo of the lima bean with different compounds. Glucose and the other sugars have caused growth, while starch or Hopkins' plant food solution failed to cause any appreciable stimulation. Also it has been shown that cotyledons which give no reaction for reducing sugars cause no growth, while these same cotyledons with glucose added give a good growth. It has been shown further that the cotyledons of beans that have germinated and contain reducing sugars also support the growth of an isolated embryo.

In summing up the results, it would appear that where glucose or a carbohydrate giving a hexose on hydrolysis is present, growth is obtained. When these substances are absent no growth results. It is also shown that the dry bean does not contain the plant food necessary for the growth of its own embryo, but that the green cotyledons of a germinated bean do contain the plant food necessary for normal growth.

We hope that other experiments which are now in progress, especially a study of the growth of the isolated embryo of the persimmon seed, will throw more light on this subject.

FETAL ATHYROSIS.

A STUDY OF THE IODINE REQUIREMENT OF THE PREGNANT SOW.

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(Received for publication, November 2, 1916.)

It has been known for a number of years that a large percentage of the sows in some sections of Montana have given birth to hairless and otherwise defective young. Many of these pigs are born dead; others die in an hour or two and very few live more than 24 to 36 hours. The resulting loss in Montana amounts to about 1,000,000 young pigs annually. While the loss is heaviest among swine, there are numerous cases among sheep and occasionally among cattle and horses.

The Affected Districts.

Losses of the same nature have been reported from western North Dakota and South Dakota, Washington,[1] Minnesota, and western Canada. In Montana, the affected area is sharply defined in some instances, less so in others. In many instances the rancher can save his pig crop by moving his pregnant sows a mile or two out of the affected district during the gestation period. Frequently the affected district is a narrow creek bottom, half a mile wide, while the bench land on either side is unaffected. 90 per cent at least of the losses in Montana occur in the drainage system of the Yellowstone River between 109° longitude and the eastern border of the state. This affected area is from 75 to 150 miles wide and is not sharply defined, but all through this region are unaffected spots, a ranch, several ranches, or a whole community, where hairless pigs have never occurred and large numbers of hogs are raised.

[1] *Washington Exp. Station, 25th Ann. Rep.*, 1915, 42.

The Affected Pigs.

The sow carries the affected pigs to the full term of the gestation period. Many ranchers report that the pregnant sows carry the pigs 4 to 7 days longer than the average period. The affected pigs are of full size, occasionally larger than normal. Born of apparently normal sows, they are strikingly weak and low in vitality. The most marked thing in the appearance of a typical specimen is the absence of hair. Except for a few tactile hairs on the nose and a few around the eyes, the skin is smooth, shiny, and bald. The hairless condition is very variable, from almost absolutely hairless to a thin coat, and through all gradations to the normal. The skin, particularly around the shoulders, is thick and feels pulpy. On making an incision the skin is seen to be $\frac{1}{2}$ to $\frac{3}{4}$ of an inch in thickness. It is semitransparent and seems edematous, but no fluid escapes on incision. The hoofs are thin-walled, short, brittle, and plainly in an undeveloped condition. The heart, in every case examined, has a persistent foramen ovale. The thyroid is dark red, sometimes almost black, and presents a most constant enlargement which varies only in proportion to the acuteness of the malady. A histological examination of the thyroid shows a uniform hyperplasia and a distention of the blood vessels.

Examination of the Thyroid.

The thyroid was separated from all connective tissue and washed in a gentle stream of water for about 15 minutes; gentle pressure was used to remove as much blood as possible. It was placed between filter papers to remove adhering moisture, and weighed, then cut into fine shreds, dried, and weighed again. The iodine content and the iron content in the moisture-free residue were determined, the iodine content by the Hunter method[2] and the iron content by filtering the dissolved melt of the iodine determination and then estimating the amount of iron, of the insoluble residue, by the thiocyanate colorimetric method.

[2] Hunter, A., *J. Biol. Chem.*, 1909–10, vii, 321.

TABLE I.

Thyroid Glands of Unaffected 1 Day Old Pigs.

No.	No. of glands.	Average weight of fresh glands.	Dry matter in fresh glands.	Iodine in desiccated glands.	Iron in desiccated glands.
		gm.	*per cent*	*per cent*	*per cent*
305	44	0.175	20.8	0.095	None.
316	4	0.208	20.9	0.214	"
318	3	0.23	18.4	0.236	"
321	3	0.27	24.2	0.064	"

TABLE II.

Thyroid Glands of Affected Pigs.

No.	Condition.	No. of glands.	Average weight of fresh glands.	Dry matter in fresh glands.	Iodine in desiccated glands.	Iron in desiccated glands.
			gm.	*per cent*	*per cent*	*per cent*
306	Hairless.	36	0.88	17.85	0.0012	0.1
307	Slightly haired, lived 2 days.	2	1.45	16.1	0.0152	0.085
308	Hairless.	3	1.1	18.3	0.0063	0.1
313	Hairless. 6 pigs living in same litter.	1	6.5	15.8	Trace.	0.08
314	"	1	2.9	18.0	0.0001	0.05
315	"	1	1.17	17.7	0.0001	0.05
317	Fairly well haired.	2	0.44	19.7	0.054	None.
319	Hairless.	1	0.64	17.35	0.00864	0.315
320	"	1	3.65	17.5	0.0047	0.15
322	Thin-haired.	1	0.7	22.3	0.015	None.
323	Fairly well haired, lived 3 days, 8 in litter.	2	1.52	15.6	0.00425	"
324	Hairless.	1	0.9	28.05	0.00444	"
325	"	2	1.59	17.55	0.00222	0.125
326	"	4	1.53	18.85	0.009	Trace.
335	"	2	0.61	18.75	0.00525	0.15
336	Skin smooth and shiny, hairless.	8	0.61	19.7	0.003	0.1
337	Hairless. Dark gland.	2	2.6	17.15	0.00135	0.112
338	" Light "	1	3.1	14.0	0.00188	Trace.

The results show that the glands of the affected pigs are abnormally large and the iodine content is extremely low in comparison with those from normal pigs, and that in general the iodine content varies inversely with the degree of hairlessness of the skin. They also show that there is a large accumulation of iron in the glands of the affected pigs. The iron content of the dried blood of the affected pigs averaged 0.2 per cent. If the accumulation of iron was due to an accumulation of a corresponding amount of blood, the greater part of the gland would be composed of blood and there would be a general hemorrhage into the follicles. The histological examination shows that the latter is not probable, and as the greater part of the blood has been removed by the washing of the gland it does not seem possible that the accumulation of iron is due to an accumulation of a corresponding amount of blood.

The Probable Sources of the Malady. A Transmissible Disease (?).

The possibility of contagious abortion was considered. Blood samples were drawn from sows within a week after producing hairless pigs, but no reaction was obtained to the complement fixation test for contagious abortion. Repeated attempts to isolate the bacillus of contagious abortion failed, though material was taken from the placenta and from the stomach contents of the hairless pigs. Further search for an infective agent was not undertaken because the field work strongly indicated that something other than transmissible disease was the cause of the malady. A rancher in one year may lose 95 per cent of his pigs and the next year with the same breeding stock, the same ranch conditions and feed, and water from the same source, raise entirely normal pigs. With the same environment some litters are hairless and others normal; in the same litter are affected and unaffected pigs. In some specific small affected areas, the sows that remain there throughout the gestation period always produce hairless pigs, while those sows that are there for only a part of the gestation period and are then removed to an unaffected area always produce normal pigs. All these facts tend to eliminate transmissible disease as a possible factor.

A Toxic Substance, Present in the Soil, Feed, or Water (?).

A considerable part of the affected areas contains a great deal of alkali, and many stockmen have asserted that alkali was the cause of the malady, but a large number of consistently normal pigs are raised in alkali districts in other parts of the state. Likewise, as alfalfa grows luxuriantly in the affected areas, and in many cases the sows have nothing else to eat, it has been stated that alfalfa would produce hairlessness in young pigs. But in many other sections of the state alfalfa is the principal food for hogs and no hairless pigs occur. In all cases where hairless pigs have been produced, the sow has been, to all appearances, perfectly normal. Can it therefore be due to a toxic substance? Can we conceive of a toxic substance such that when ingested by the mother, it so persistently leaves her to all appearances perfectly normal, yet arrests the development of the fetal growth to such a remarkable extent—one with such a specific action that it only functions in the later stages of the intra-uterine life? The effects do not correspond to the physiological action of any known toxic substance; therefore the probability of a toxic substance being the cause of the malady appears to be very remote.

A Deficiency of Some Essential Constituent.

The apparent symptoms of the affected pigs correspond exactly to the symptoms of myxedema as described by Ord[3] and also to the symptoms occurring after the extirpation of the thyroid as described by the Reverdins,[4] Kocker,[5] and Eiselberg.[6] The hyperplasia of the thyroid varies proportionally with the acuteness of the malady. The results of Hunt and Seidell,[7] Marine,[8] and Marine and Williams[9] show conclusively that the activity of the thyroid depends quantitatively on the iodine content. As

[3] Ord, W. M., *Med.-Chir. Tr.*, 1878, xli, 57.

[4] Reverdin, J. L., and Reverdin, A., *Rev. méd. Suisse romande*, 1883, iii, 169, 233, 309.

[5] Kocker, T., *Arch. klin. Chir.*, 1883, xxix, 254.

[6] von Eiselberg, A., *Arch. klin. Chir.*, 1894, xlix, 207.

[7] Hunt, R., and Seidell, A., *Bull. Hyg. Lab., U. S. P. H.*, 47, 1909.

[8] Marine, D., *Bull. Johns Hopkins Hosp.*, 1907, xviii, 359.

[9] Marine, D., and Williams, W. W., *Arch. Int. Med.*, 1908, i, 378.

the iodine content of the glands of the affected pigs is extremely low, then the activity of thyroid must be correspondingly low. Therefore there seems strong reason to believe that the arrested fetal development of the affected pigs is due to the decreased physiological activity of the thyroid, which the author has designated as fetal athyrosis. On the above considerations an investigation into the iodine requirement of the pregnant sow and a search for the amount of available iodine in the affected districts were undertaken.

A large number of samples of agricultural products from affected and unaffected districts were examined for their iodine content. The results did not indicate anything very definite. Iodine was found in only a few of the samples collected, and in very small amounts. The analyses, however, showed that the amount of available iodine is low and that the average iodine content of samples from affected districts was lower than of samples from unaffected districts. From all results the following has been deduced: While there appears to be enough available iodine to produce the physiologically active constituents of the thyroid required by both young and adult animals, yet, more especially in the affected districts of Montana and to a certain extent over all the western regions, the available iodine is near the border line of the amount required by the pregnant animal. If certain unknown conditions are favorable, the mother may obtain the amount of iodine required for the activities both of its own and fetal glands, but if these conditions are not favorable, the maternal organism can still obtain the amount required for the activities of its own thyroid but not enough for the rapidly growing fetus. The iodine starvation of the fetus depresses the physiological activity of the fetal thyroid which causes the remarkably arrested development peculiar to this malady. This is a modification of the contention that has already been brought forward by Fenger[10] after he had examined a great number of adult and fetal glands from animals killed in Chicago. Fenger found that a large percentage of the fetal glands were abnormally large and had a very low iodine content, which he states is not the case with the adult glands. From this he contended that

[10] Fenger, F., *J. Biol. Chem.*, 1913, xiv, 404.

"the demand for iodine in the rapid fetal metabolism and growth in certain instances may exceed the available supply furnished by the pregnant animal. This supply may be sufficient for the maintenance of the maternal metabolism leaving the adult thyroid normal, but not sufficient to prevent iodine starvation and enlargement of the fetal gland."

Iodine Feeding.

Hunt and Seidell[7] say their results "show that when potassium iodide or iodoform is administered to dogs, the thyroids of the latter contain a greatly increased percentage of iodine and also are much more active physiologically."

Marine,[11] in reporting upon glandular hyperplasias of dog thyroids, says that "lack of iodine was the essential deficiency, and iodine when supplied quickly overcame the needs."

Experiment 1.—In the fall of 1915 fifteen sows from an unaffected district were divided into three pens. They were placed on an affected ranch and were bred about December 1, and kept there during the gestation period. They were fed 3 to 5 pounds daily of mixed and red clover hay and sufficient water, all products of the affected ranch. One pen was fed potassium iodide, 15 grains daily per sow, from December 15 to farrowing time; another pen was fed powdered sheep thyroid, and the remainder of the sows were used as a check.

TABLE III.

Pen.	No. of sows.	Special treatment.	No. of young pigs.	Condition of young pigs.	Losses.
1	7	Potassium iodide, 15 grains daily.....	56	Strong and vigorous.	2
2	5	Sheep thyroid, 5 grains daily.....	32	Good.....	6
3	5	No potassium iodide or sheep thyroid.....	18	Very poor, weak, dull, and no vitality.....	13

[11] Marine, *J. Infect. Dis.*, 1907, iv, 425.

The owner of the affected ranch added two sows to Pen 1. These had each produced hairless pigs in March and October, 1915. Only four of the sows farrowed in Pen 3 and they produced eighteen pigs. These were weak, wrinkled, squeaking, and puny pigs that appeared to have no chance to thrive; thirteen died in the first few days. They were not hairless but strongly resembled the survivors of a litter which contained hairless pigs. They were thin-haired, had no vitality, and the difference in appearance between these and the pigs of Pen 1 was striking, though the most careful observation could show no material difference between the sows in the two pens.

Experiment 2.—Potassium iodide, in 5 grain daily doses, was fed to twenty-eight sows on five ranches in the affected districts for the last 4 or 5 weeks of the gestation period. The sows that had already farrowed on these ranches had produced hairless pigs. Each of the twenty-eight sows produced normal pigs after receiving potassium iodide.

The object of the above experiments was to demonstrate the difference between the conditions under which hairless pigs are produced and the conditions under which normal pigs are produced. As no hairless pigs were produced in Experiment 1, it appeared, at first, as if the results of that experiment were useless, but there was such a marked difference in the vitality of the young pigs in the respective pens that the results can be considered as conclusive as if hairless pigs had been produced, especially if the intermittent nature of the malady is taken into consideration. The results, besides showing that an abundant supply of iodine counteracts fetal athyrosis, also give a striking demonstration of the beneficial effect of feeding iodine to the pregnant sow.

When iodine is administered as potassium iodide it is quickly detected in the urine but in combination with fats it is retained for a relatively long period in the system; therefore it would probably be more economical to feed iodine as the di-iodostearate obtained by saturating with iodine certain vegetable oils, *viz.*, cottonseed oil, olive oil, etc.

It is intended to recommend the feeding of iodine to the pregnant sows throughout the whole affected areas during the coming season. The wide scope of the test should produce conclusive results.

DISCUSSION.

All the evidence indicates conclusively that the malady is caused by a lack of function of the thyroid. On account of the pronounced hyperplasia of the gland it might be assumed by others that the abnormal growth was produced by some infective agent of low virulence that could not be readily detected, or by some toxic substance, as digitalin, abrin, and ricin are known to produce hyperplasia of the thyroid, and that the beneficial effect of administering potassium iodide was due to the iodide enabling the fetus to resist the infection or neutralizing the action of the toxic substance. It has been pointed out that all evidence tends to disprove these contentions; therefore in the absence of any evidence of the presence of any infective agent or toxic substance, it is most feasible to assume that the cause of the lack of function of the thyroid is due to a deficiency of iodine.

During an investigation of the effect of diet on the activity of the thyroid, Reid Hunt[12] made the hypothesis that certain diets lead to an extensive elimination of iodine of the thyroid. His results indicate that the elimination is greatest with those diets that produce the more rapid growth, eggs, milk, cheese, and various fats.

Fetal athyrosis is prevalent where the sows are fed on alfalfa and flaxseed, and it has occurred, in some cases, where the sows have received large quantities of milk. It is generally recognized that these feeds produce rapid growth and it is probable that they might lead to an abundant secretion of the thyroid and thus exhaust the thyroid of its physiologically active constituents. Therefore, while in the presence of sufficient iodine they may be excellent feeds for the pregnant animal, yet when the amount of available iodine is low they may cause this malady to become more acute. The fact that some of the specimens that were extremely bald were much larger than normal would appear to support this contention.

Seidell and Fenger[13] found that there was a great seasonal variation in the iodine content of the thyroids of sheep, cattle, and hogs killed in Chicago. The iodine content was at a maxi-

[12] Hunt, R., *Bull. Hyg. Lab., U. S. P. H.*, 69, 1910.

[13] Seidell, A., and Fenger, F., *J. Biol. Chem.*, 1912–13, xiii, 517.

mum in the summer, dropped rapidly during the winter, and rose to a maximum again the following summer. Their results show that the iodine content of the glands of hogs is lowest from December to March, the period of the intra-uterine life of the spring litters, and that the yearly iodine curve for hogs is much higher than the curves for sheep and cattle.

It has been noticed that pigs born in March and April are much more frequently affected with fetal athyrosis than those born in May and June, and fall pigs are most frequently normal even in the badly affected districts, which indicates that the seasonal variation exerts a great influence upon the acuteness of this malady, and as the yearly iodine curve for hogs is much higher than the curves for sheep and cattle, a deficiency of the thyroid constituent would cause the offspring of hogs to be more susceptible to fetal athyrosis than those of sheep and cattle. This is in accordance with the well established evidence of the occurrence of the malady.

The results of Martin[14] have shown that composite samples of thyroids, collected throughout the year from sheep slaughtered at Newcastle-on-Tyne, England, varied comparatively little in their iodine content. A striking feature of the results was the uniformly small size of the glands and the relatively high per cent of iodine.

The breeders in Great Britain have won a unique reputation for the excellent livestock that they produce and fetal athyrosis has never been known to occur there. The above results therefore indicate that the shortage of iodine is more acute in North America than in Great Britain. When all the results are correlated it appears feasible to assume that if the pregnant animals on this continent were given more iodine, especially during the winter months, the young that they produce would be more healthy and more vigorous and the number of weak and defective young animals that are produced annually would be greatly reduced.

It has been generally contended by others that the lack of growth of the epidermal appendages in myxedema and after extirpation of the thyroid does not indicate a direct relation

[14] Martin, N. H., *Brit. and Colonial Druggist*, 1912, lxii, 99.

between the thyroid and the production of those appendages, but is due to inhibition by the toxic substances of metabolism which have not been neutralized by secretion of the thyroid. There is every evidence that the development of the epidermal appendages is arrested, in fetal athyrosis, in the later stages of the intra-uterine life when the fetal blood is oxygenated by the maternal organism, and it can be assumed that any toxic substance in the fetal blood would be neutralized at the same time by the maternal organism. Therefore it does not seem possible that the lack of growth of the epidermal appendages as found in fetal athyrosis and probably also after extirpation of the thyroid and with myxedema is due to inhibition by toxic substances of metabolism and growth. While it may be feasible to assume that the maternal organism would neutralize the toxic substances of the fetal blood, it is not probable that the maternal thyroid could give the required assistance for the proper development of the fetus.

It has been pointed out that the thyroids of the affected pigs are a very dark red color and that there is an accumulation of iron in the gland. This accumulation of iron is significant as myxedema is accompanied by anemia and after extirpation of the thyroid there is a rapid decrease of red corpuscles.

CONCLUSIONS.

An iodine deficiency during the gestation period causes a lack of function and hyperplasia of the fetal thyroid, resulting in an arrested development of the fetus.

If more iodine were fed to the pregnant animals in large sections of this continent, especially during the winter months, the young that they produce would be more healthy and more vigorous and the large number of weak and defective young animals that are produced annually would be greatly reduced.

Fetal athyrosis presents strong evidence that there is a direct relation between the physiologically active constituents of the thyroid and growth of the epidermal appendages.

An abundant secretion of the fetal thyroid, during the later stages of the intra-uterine life, is essential for the normal development of the fetus.

STUDIES IN CARBOHYDRATE METABOLISM.

XIV. THE INFLUENCE OF ALKALI ADMINISTRATION UPON BLOOD SUGAR CONTENT IN RELATION TO THE ACID-BASE-PRODUCING PROPERTIES OF THE DIET.*

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The influence of acid and alkali upon carbohydrate metabolism with respect to glycosuria and blood sugar content has recently received considerable attention. In a discussion of the subject, it seems desirable to differentiate between the results obtained by administration of acid or alkali to normal animals and those yielded when the sugar-regulating mechanism has been temporarily disturbed.

Elias demonstrated that the introduction of hydrochloric acid (0.25 N) into dogs and rabbits leads to hyperglycemia and glycosuria. Underhill (1916, *a*) found that the subcutaneous injection of calcium salts (lactate and chloride), which under disturbed conditions of carbohydrate metabolism have an effect analogous to hydrochloric acid, is without significant influence upon the blood sugar content of normal rabbits; and that the withdrawal of calcium by the introduction of sodium phosphate will usually, although not invariably, produce in normal rabbits a condition of hypoglycemia. This investigator showed also (1916, *b*) that the intravenous injection of sodium carbonate into normal animals may induce a decided though transient fall in blood sugar content. Marked hypoglycemia is indicated in slightly more than one-half the instances reported.

Recently Macleod and Fulk found that the injection of sodium carbonate solution or of hydrochloric acid, at intervals, into the portal vein during the continuous injection of a glucose solution into another branch of the same vein has no effect upon the blood sugar. But when glucose is injected at a rate below the tolerance limit with sufficient alkali to reduce distinctly the hydrogen ion concentration of the blood of the portal vein and vena cava, the blood sugar does not rise to such a degree as it does when the glucose solution is neutral or acid in reaction.

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* The data in this and the six papers following are taken from the dissertation presented by Louise McDanell for the degree of Doctor of Philosophy, Yale University, 1917.

The influence of the introduction of acid or alkali into animals with a defective sugar-regulating mechanism has been studied more extensively. Pavy and Godden gave intravenously a 3 per cent solution of anhydrous sodium carbonate to cats exhibiting glycosuria in consequence of the administration of chloroform. They observed a fall in the intensity of the glycosuria within 15 minutes after the injection was begun, the urine becoming sugar-free at the end of 30 minutes. It was suggested by them that the inhalation of chloroform may produce a condition of acidosis which leads to an abnormal conversion of glycogen into sugar, and hence to glycosuria. Elias and Kolb studied the influence of sodium carbonate upon glycosuria, hyperglycemia, and sugar tolerance in the "hunger diabetes" of young dogs, and found that the alkali reduces the glycosuria and glycemia. That this "hunger diabetes" is a condition of acidosis is indicated by the lowered carbon dioxide content of the blood and alveolar air. Murlin and Kramer (1913) showed that after removal of the pancreas, sodium carbonate introduced into the blood stream of the dog causes a diminution of the sugar excretion. Later experiments by the same authors (1916) indicated: first, that sodium bicarbonate and potassium bicarbonate administered by stomach tube may be without immediate effect upon the glycosuria and hyperglycemia of the depancreatized dog; second, that an alkali bicarbonate given by mouth to a fasting depancreatized dog may even cause the reappearance of glucose in the urine after it has been "starved out;" third, that the normal anhydrous salt of sodium, Na_2CO_3, may, on the contrary, reduce the sugar in the urine materially, when given by mouth, and when given by vein invariably does so, especially when added to Ringer's or Locke's solution to the amount of about 1 per cent; and fourth, that dilute hydrochloric acid given by mouth or subcutaneously to the depancreatized dog has an effect opposite to that of alkali, increasing the sugar in the urine without affecting materially the nitrogen elimination, and without causing any effect upon the blood sugar.

It was shown by Underhill and Blatherwick that in thyreoparathyroidectomized animals, the blood sugar content may be greatly diminished previous to the onset of tetany. At this period, Wilson, Stearns, and Thurlow, and Wilson, Stearns, and Janney found that a condition of alkalosis exists, the duration of which corresponds fairly well with the interval of low blood sugar observed by Underhill and Blatherwick. The tetany is relieved and the blood sugar restored to normal by the introduction of calcium. The intravenous administration of hydrochloric acid relieves the tetany, but apparently its effect upon the blood sugar has not been investigated. It seems probable that the condition of alkalosis may be responsible for the hypoglycemia.

It was first shown by Underhill (1911) that the subcutaneous injection of hydrazine sulfate invariably leads to hypoglycemia in dogs, and frequently in rabbits. Later MacAdam called attention to the fact that, provided the dose of hydrazine is sufficiently large, rabbits also always exhibit hypoglycemia. Underhill and Baumann recently demonstrated

that, in the dog, during this period of hypoglycemia, the hydrogen ion concentration of the urine is greatly depressed, even to the point of marked alkalinity.

Underhill (1916, *b*) found that sodium carbonate, if administered at suitable intervals previous to epinephrine, significantly reduces the hyperglycemia and glycosuria incident to injections of that drug. Calcium, which Wilson and his coworkers showed to exert an acid-like action at times in the body, exhibits (Underhill, 1916, *a*) an effect opposite to that of sodium carbonate, causing an increased elimination of sugar in the urine, and altering the character of the curve of epinephrine hyperglycemia. The withdrawal of calcium, by the injection of sodium phosphate prior to the administration of epinephrine, reduces the sugar level and shortens the period of hyperglycemia, and frequently diminishes the output of sugar in the urine, in comparison with conditions brought about by epinephrine alone. It has been shown by Macleod and Fulk that there is a distinct decrease in the blood sugar in animals in which there is hyperglycemia (etherization and operative manipulation in sugar-fed dogs), when alkali (Na_2CO_3) is injected intravenously in sufficient amount to lower the hydrogen ion concentration of the blood. Underhill (1916, *c*) reported the disappearance of glycosuria in a very severe case of human diabetes, following the ingestion of large doses of sodium bicarbonate.

The experiments detailed in the present paper were undertaken for the purpose of determining the factors responsible for the inconstant production of hypoglycemia in normal rabbits after the intravenous injection of sodium carbonate, as reported by Underhill (1916, *b*). The significance of the diet, with respect to its potential acid- or base-producing properties, has received special attention.

Methods.

The experiments were performed upon healthy, full-grown rabbits. Blood was obtained from the ear vein, the sample weighed, and the blood sugar estimated by the method of Lewis and Benedict.[1, 2] After a sample of blood had been taken for the normal blood sugar, sodium carbonate in 0.5 per cent solution was introduced, under pressure, into the ear vein, 2 to 5 minutes being required for the injection. Samples of blood for the determination of sugar were taken 30, 60, and 90 minutes thereafter. Allowance was not made for dilution of the blood, as other experiments in this laboratory[3] have demonstrated that,

[1] McDanell, L., *J. Lab. and Clin. Med.*, 1916, i, 804.

[2] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

[3] Bogert, L. J., Underhill, F. P., and Mendel, L. B., *Am. J. Physiol.*, 1916, xli, 189.

under the conditions here maintained, the blood volume returns to normal in less than 30 minutes, the interval allowed before the first sample was taken.

No difficulty was experienced in these experiments, or elsewhere, on account of the "emotional glycosuria" described by Cannon, Shohl, and Wright.[4] Allen[5] accounts for the variations in the blood sugar of the rabbit, as given by various authors, by the statement: "The rabbit is an animal subject to emotional and traumatic hyperglycemia." Later he explains the apparent low tolerance of the rabbit for glucose, in some instances, by saying: "Traumatic, emotional, or other disturbing influences are probably the explanation, in a highly erratic and uncertain animal." The experience in this laboratory, where many rabbits are used, leads to an opposite conclusion. Rabbits are usually quiet, docile, and seemingly unconcerned about any ordinary treatment accorded them. Even when apparently excited, there has been no noticeable effect upon the blood sugar.

The diets of these rabbits were the same as those used by Underhill[6]—whole oats and cracked corn for the acid-forming diet, carrots for the base-forming diet, and a mixture of these for the "mixed" diet.

The results are shown in Tables I, II, and III. From these it may be seen that, with the single exception of Rabbit 17, there was no marked diminution of the blood sugar following the injection of alkali. The initial blood sugar of Rabbit 17 was much higher than that of any other rabbit which was used, and, after the drop of one-third in the first half hour, it was still above our average for normal rabbits. The further wide variations in the blood sugar in subsequent periods would tend to confirm the belief that Rabbit 17 is not to be considered a normal animal. While there are a few instances of an appreciable augmentation of blood sugar content following the injection of the sodium carbonate (Rabbits 18, 19, 22), the increase is too slight to be attributable to the alkali, in view of the large number of cases which showed no significant change. The hyperglycemia may be

[4] Cannon, W. B., Shohl, A. T., and Wright, W. S., *Am. J. Physiol.*, 1911–12, xxix, 280.

[5] Allen, F. M., Studies concerning Glycosuria and Diabetes, Boston, 1913.

[6] Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127.

accounted for in Rabbit 22 by the prostration incident to the injection. The few cases of prostration in our experience have all been accompanied by a marked hyperglycemia.

The data presented indicate that the injection of sodium carbonate is as ineffective in altering the blood sugar content of normal rabbits maintained upon either acid-forming or base-forming diets as it is in animals upon a mixed diet.

TABLE I.

The Influence of Intravenous Injections of 0.5 per Cent Sodium Carbonate upon the Blood Sugar Content of Rabbits Maintained upon a Mixed Diet.

Date.	Rabbit.	Weight.	Volume of sodium carbonate injected.	Blood sugar (in percentages).			
				Normal.	Min. after injection.		
					30	60	90
		gm.	*cc.*				
March 7.....	15	2,200	125	0.13	0.132	0.14	0.122
" 7.....	16	2,060	125	0.132	0.133	0.13	0.133
" 11.....	17	1,940	125	0.18	0.12	0.176	0.115
" 11.....	18	1,980	125	0.14	0.20	0.188	0.19
" 13.....	19	2,800	125	0.12	0.149	0.118	0.144
" 13.....	20	2,000	100	0.11	0.12	0.12	0.117
" 15.....	21	2,360	125	0.119	0.12	0.115	0.124
" 15.....	22*	2,480	120	0.116	0.154	0.164	0.142
" 17.....	23	2,680	140	0.11	0.117	0.137	0.136
" 17.....	24	2,300	125	0.131	0.124	0.12	0.143

* Prostrated by injection.

TABLE II.

The Influence of Intravenous Injections of 0.5 per Cent Sodium Carbonate upon the Blood Sugar Content of Rabbits Maintained upon an Acid-Forming Diet.

Date.	Rabbit.	Weight.	Volume of sodium carbonate injected.	Blood sugar (in percentages).			
				Normal.	Min. after injection.		
					30	60	90
		gm.	*cc*				
May 3.....	27	1,420	120	0.119	0.137	0.15	0.139
" 3.....	29	1,900	120	0.115	0.13	0.13	0.127
" 5.....	30	1,660	123	0.11	0.117	0.119	0.123
" 5.....	31	1,760	110	0.117	0.13	0.13	0.14

TABLE III.

The Influence of Intravenous Injections of 0.5 per Cent Sodium Carbonate upon the Blood Sugar Content of Rabbits Maintained upon a Base-Forming Diet.

Date.	Rabbit.	Weight.	Volume of sodium carbonate injected.	Blood sugar (in percentages).			
				Normal.	Min. after injection.		
					30	60	90
		gm.	*cc.*				
May 10.....	29	2,000	123	0.102	0.115	0.11	0.11
" 10.....	32	1,500	120	0.12	0.112	0.126	0.135
" 11.....	33	2,080	120	0.104	0.11	0.115	0.12
" 11.....	34	1,760	120	0.113	0.107	0.107	0.109
" 12.....	35	2,140	120	0.104	0.104	0.113	0.111
" 12.....	36	1,540	60	0.11	0.115	0.11	0.114

CONCLUSIONS.

In new experiments upon the influence of sodium carbonate on the blood sugar content of normal rabbits on a mixed diet, hypoglycemia has invariably failed to manifest itself.

The variable potency of sodium carbonate in the production of hypoglycemia, as reported by Underhill, cannot be explained by variations in the acid-base-producing possibilities of the diet, inasmuch as wide ranges in these directions apparently play a small part in influencing the blood sugar content of normal rabbits.

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STUDIES IN CARBOHYDRATE METABOLISM.

XV. THE INFLUENCE OF ACID-FORMING AND BASE-FORMING DIETS UPON BLOOD SUGAR CONTENT.

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As the result of a study, by Sherman and Gettler, of the ash of foods, emphasis has been laid upon the striking differences of foodstuffs with respect to their property of yielding an ash with a preponderance of either acid or basic elements. They demonstrated that fruits and vegetables yield a basic ash, and that cereals, meat, and fish give an acid ash. It was also pointed out by these investigators that the acid-forming or base-forming character of ingested food plays a significant rôle in the regulation of urinary composition. Thus, they found an augmentation in the ammonia excretion equivalent to as much as one-third of the increased potential acid in the diet. A still greater proportion of this acid was represented by the increased acidity of the urine.

Blatherwick extended the work of Sherman and Gettler with respect to the relation of the urinary reaction to the character of the diet. He found that in general the reaction of the urine varies with that of the ash constituents of the diet. Exceptions to the rule were noted with plums, prunes, and cranberries, which yield an acid urine. The cause for this unexpected result is ascribed to their content of benzoic acid, which is incompletely oxidized in the body and excreted as hippuric acid. Underhill, in a study of creatine metabolism, showed that in rabbits, which normally excrete an alkaline urine, a diet of oats and cracked corn gives rise to a urine of high acidity, while a diet of carrots increases the alkalinity beyond the normal.

In an investigation of the influence of acid and alkali upon carbohydrate metabolism, it was deemed desirable to determine whether any influence would be exerted upon blood sugar content by differences in the acid-base character of the diet. The detailed experiments follow.

Methods.

The experiments were made upon normal, full-grown rabbits. The diets were the same as those used by Underhill—oats and cracked corn for the acid-forming diet, carrots for the base-forming diet, and a mixture of these for the "mixed" diet. Water was offered several times a day. Blood sugar was determined, usually in the morning and afternoon, according to the method of Lewis and Benedict.[1, 2] For the estimation of hydrogen ion concentration in the urine, the procedure of Henderson and Palmer[3] was employed, and the results are expressed in terms of the logarithmic notation of Sörensen used by them. On account of the strong alkalinity of the urine from the carrot and mixed diets, hydrogen ion concentration was determined only during the periods of the grain diet.

Tables I and II show the results for rabbits which were under observation for about 6 weeks. The results for rabbits for which the experimental period was shorter are given in Tables III to VI.

The most striking fact brought out by these data is the constancy of the values for blood sugar, notwithstanding the wide variations in the hydrogen ion concentration of the urine. Evidently the sugar-regulating mechanism of a normal rabbit is not significantly disturbed by such variations in acidity as occurred here; or, expressed differently, its power of compensation is sufficiently great to enable it to handle these amounts of acid without interference with the acid-base equilibrium of its body.

Attention may be called to the cases of greatest variation in the blood sugar content. Rabbit 25 showed a blood sugar value of 0.085 per cent on March 27 and of 0.084 per cent on April 5, while with Rabbit 26 there was a value of 0.085 per cent on March 27, and of 0.076 per cent on April 6. These low values occurred during the acid-forming diet, only a small portion of which was eaten, and also after the rabbits, both of which were small, had lost considerable weight. The cases of highest blood sugar, Rabbit 26, 0.161 per cent on April 24, and Rabbit 30, 0.162 per cent on April 19, as well as other high values, were obtained upon

[1] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

[2] McDanell, L., *J. Lab. and Clin. Med.*, 1916, i, 804.

[3] Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, xiii, 393.

the base-forming rations. This diet the rabbits relished; usually they ate all that was given them, and upon it maintained their weight or even gained. Other experiments in this series[4] show that rabbits store very little glycogen upon an acid-forming diet when the intake is as small as it was with the animals under discussion. It is therefore possible that the variations noted in the blood sugar content may be related to dietary factors other than those concerned with the acid-base properties.

TABLE I.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content. Rabbit 25.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Mixed diet.*						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
March 20...		1,700	0.119	0.135		
" 21...			0.122	0.104	75	Alkaline.
" 22...	Grain 30 Carrots 125		0.108	0.104	44	"
" 23...	Grain 39 Carrots 120		0.098	0.114	45	"
Acid-forming diet.†						
March 24...	40	1,600	0.114	0.121	18	Alkaline.
" 25...	35		0.123		25	"
" 26...	30		0.10		25	"
" 27...	40	1,500	0.089	0.085	25	"
Base-forming diet.‡						
March 28...	430		0.122	0.13	90	Alkaline.
" 29...	500	1,500	0.129	0.125	275	"
" 30	500		0.113		375	"
" 31...	500	1,400	0.121	0.116	385	"

* Corn and oats, 50 gm., carrots, 250 gm.

† Corn and oats, 100 gm.

‡ Carrots, 500 gm.

[4] McDanell, L., and Underhill, F. P., *J. Biol. Chem.*, 1917, xxix, 255.

TABLE I—*Concluded.*

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Acid-forming diet.†						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
April 1.....	57			0.114	15	Alkaline.
" 2.....	40	1,400	0.119		25	
						H ion.
" 3.....	52		0.111	0.117	15	5.5
" 4.....	43	1,440	0.092	0.098	20	5.7
" 5.....	55		0.105	0.084	20	5.7
" 6.....	55	1,460	0.10	0.106	20	5.7
" 7.....	60		0.10	0.098	10	5.5
" 8.....	57	1,440	0.102	0.087	30	5.8
" 9.....	60		0.097		25	5.3
" 10....		1,440	0.106	0.098	50	5.8
Base-forming diet.‡						
						H ion.
April 11.....	488		0.113	0.10	115	6.7
" 12.....	500	1,500	0.096	0.101	260	6.9
" 13.....	500		0.099		285	7.38
" 14.....	500	1,460	0.109	0.11	260	Alkaline.
" 15. ...	500		0.126	0.113	330	"
" 16.....	500	1,460	0.115		315	"
" 17.....	455		0.132	0.113	245	"
" 18.....	500	1,500	0.107	0.106	360	"
" 19.....	500		0.107	0.118	245	"
" 20.....	500	1,520	0.116	0.101	285	"
" 21.....	500		0.11	0.116	265	"
" 22.....	500	1,520	0.106	0.121	325	"
" 23. ...	500		0.124		365	"
" 24.....	500		0.133	0.135	320	"
Acid-forming diet.†						
April 25.....	52		0.117	0.123	75	Alkaline.
						H ion.
" 26.....	50	1,520	0.097	0.106	45	5.3
" 27.....	30		0.116		36	4.9
" 28.....	25	1,500	0.112	0.119	26	4.7
" 29.....	15		0.118	0.097	25	5.3
" 30.....	10	1,440	0.113		15	5.3

TABLE II.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content. Rabbit 26.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Mixed diet.*						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
March 20...		2,000	0.128	0.119		
" 21...			0.128	0.108	140	Alkaline.
" 22...			0.115	0.112	108	"
" 23...	Grain 50 Carrots 250		0.121		108	"
Acid-forming diet.†						
March 24...	55	1,840	0.11	0.115	64	Alkaline.
" 25...	52		0.108		80	"
						H ion.
" 26...	10		0.097		130	7.38
" 27...	8	1,560	0.085	0.098	155	7.43
Base-forming diet.‡						
March 28...	410		0.121	0.123	230	Alkaline.
" 29...	500	1,720	0.129	0.121	385	"
" 30...	500		0.115		315	"
" 31...	500	1,720	0.12	0.11	400	"
Acid-forming diet.†						
April 1.....	50			0.121	110	Alkaline.
" 2.....	60	1,680	0.117		95	
						H ion.
" 3.....	46		0.129	0.103	80	6.7
" 4.....	60	1,660	0.128	0.123	75	6.5
" 5.....	53		0.117	0.092	80	6.8
" 6.....	45	1,640	0.076	0.093	60	6.3
" 7.....	41		0.108	0.103	55	5.7
" 8.....		1,600	0.111	0.117	55	5.7
" 9.....	40		0.097		60	5.5
" 10.....			0.104	0.099	45	5.3

* Corn and oats, 50 gm.; carrots, 250 gm.

† Corn and oats, 100 gm.

‡ Carrots, 500 gm.

TABLE II—*Concluded.*

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Base-forming diet.‡						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
April 11.....	500		0.095	0.10	175	6.8
" 12.....	500	1,620	0.107	0.093	400	7.38
" 13.....	500		0.112	0.119	285	Alkaline.
" 14.....	500	1,680	0.108	0.106	385	"
" 15.....	500		0.113	0.148	320	"
" 16.....	500	1,660	0.107		505	"
" 17.....	500		0.109	0.136	305	"
" 18.....	500	1,800	0.098	0.127	445	"
" 19.....	500		0.113	0.126	420	"
" 20.....	500	1,880	0.133	0.132	275	"
" 21.....	500		0.129	0.158	285	"
" 22.....	600	1,800	0.134	0.128	395	"
" 23.....	500		0.132		360	"
" 24.....	500		0.114	0.161	450	"
Acid-forming diet.†						
April 25.....	80		0.114	0.133	95	Alkaline.
" 26.....	93	1,780	0.124	0.111	38	
						H ion.
" 27.....	63		0.13		35	4.7
" 28.....		1,760	0.12	0.112		
" 29.....	60		0.122	0.128	36	4.7
" 30.....		1,740	0.138			

TABLE III.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content. Rabbit 27.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Base-forming diet.*						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
April 14.....		1,820				
" 15.....	460		0.114	0.104	210	Alkaline.
" 16.....	500	1,900	0.112		325	"
" 17.....	500		0.114	0.102	320	"
" 18.....	500	1,900	0.098	0.094	345	"
" 19.....	500		0.114	0.117	250	"
" 20.....	500	1,860	0.106	0.116	295	"
" 21.....	500		0.124	0.107	255	"
" 22.....	410	1,800	0.113	0.11	295	"
" 23.....	500		0.116		300	"
" 24.....	440	1,780	0.11	0.123	280	"
Acid-forming diet.†						
April 25.....			0.115	0.099	55	Alkaline. H ion.
" 26.....	33	1,640	0.11	0.10	29	5.85
" 27.....	42		0.12		35	5.3
" 28.....		1,620	0.134	0.107		
" 29.....	60		0.114	0.095	56	5.7
" 30.....	20	1,540	0.118		40	4.7
May 1.....	5		0.13		35	4.7

* Carrots, 500 gm.

† Corn and oats, 100 gm.

TABLE IV.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content. Rabbit 29.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Base-forming diet.*						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
April 14.....		1,980				
" 15.....	402		0.117	0.11	240	Alkaline.
" 16.....	500	2,020	0.108		245	"
" 17.....	500		0.109	0.096	325	"
" 18.....	500	2,100	0.108	0.11	340	"
" 19.....	500		0.127		365	"
" 20.....	500	2,000	0.118	0.106	375	"
" 21.....	500		0.133	0.11	280	"
" 22.....	500	2,080	0.118	0.114	280	"
" 23.....	500		0.123		420	"
" 24.....	500	2,020	0.127	0.127	270	"
Acid-forming diet.†						
April 25.....	80		0.116	0.112	45	Alkaline.
						H ion.
" 26.....	90	1,960	0.115	0.104	30	6.3
" 27.....	65		0.11		15	5.85
" 28.....	65	2,000	0.11	0.104	20	5.7
" 29.....	40		0.106	0.106	29	5.85
" 30.....	23	1,940	0.116		26	5.7
May 1.....	20		0.119		22	5.7

* Carrots, 500 gm.

† Corn and oats, 100 gm.

TABLE V.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content. Rabbit 30.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Base-forming diet.*						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
April 14.....		1,920				
" 15.....	500		0.136	0.106	350	Alkaline.
" 16.....	500	1,820	0.132		400	"
" 17.....	500		0.123	0.123	310	"
" 18.....	500	1,920	0.135	0.125	340	"
" 19.....	500		0.162	0.137	210	"
" 20.....	500	1,980	0.143	0.123	310	"
" 21.....	500		0.125	0.123	370	"
" 22.....	500	1,880	0.125	0.125	390	"
" 23.....	500		0.128		405	"
" 24.....	500	1,820	0.108	0.10	400	"
Acid-forming diet.†						
April 25.....	76		0.118	0.116	55	Alkaline.
						H ion.
" 26.....	75	1,800	0.11	0.127	45	5.1
" 27.....	50		0.105		55	4.7
" 28.....	45	1,720	0.106	0.10	44	4.7
" 29.....	18		0.107	0.102	30	4.7
" 30.....	30	1,680	0.108		35	4.7
May 1.....	45		0.10		34	4.9
" 2.....	55	1,720	0.107	0.09	30	5.1
" 3.....	57		0.11		40	4.9
" 4.....	50		0.109		22	5.1
" 5.....	28	1,660	0.11		27	4.9

* Carrots, 500 gm.

† Corn and oats, 100 gm.

TABLE VI.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content. Rabbit 31.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Base-forming diet.*						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
April 16.....	500	2,040	0.102		290	Alkaline.
" 17.....	500		0.109	0.105	370	"
" 18.....	500	2,040	0.105	0.103	390	"
" 19.....	500		0.107	0.112	275	"
" 20.....	500	1,960	0.127	0.115	370	"
" 21.....	500		0.119	0.118	290	"
" 22.....	500	2,020	0.12	0.111	370	"
" 23.....	500		0.111		320	"
" 24.....	500	2,060	0.114	0.123	370	"
Acid-forming diet.†						
April 25.....	75		0.125	0.099	85	Alkaline. H ion.
" 26.....		2,080	0.109	0.101	85	6.3
" 27.....	138		0.106		8	6.9
" 28.....	35	2,040	0.11	0.098	105	5.3
" 29.....	28		0.112	0.107	125	5.3
" 30.....	33	1,940	0.105		78	4.7
May 1.....	35		0.109		90	4.7
" 2.....	18	1,840	0.117		120	4.7
" 3.....	60			0.114	55	4.7
" 4	5		0.128		67	4.7
" 5.....	20	1,760	0.117		75	4.7

* Carrots, 500 gm.

† Corn and oats, 100 gm.

TABLE VII.

The Average Figures for Blood Sugar Content of Rabbits Maintained upon Different Diets.

Rabbit.	Acid-forming diet.	Base-forming diet.
	per cent	*per cent*
25 Period 1	0.105	0.122
" 2	0.102	0.113
" 3	0.111	
26 Period 1	0.102	0.12
" 2	0.108	0.121
" 3	0.123	
27	0.112	0.111
29	0.111	0.115
30	0.108	0.127
31	0.11	0.113
General averages	**0.109**	**0.117**

CONCLUSIONS.

In a normal rabbit the blood sugar content is not significantly changed by variations in the acid-base content of the diet, which are sufficient to cause a marked change in the hydrogen ion concentration of the urine. These results are in agreement with those of the preceding paper, which indicate that the blood sugar content of a normal rabbit is not usually appreciably affected by intravenous injections of dilute alkali.

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STUDIES IN CARBOHYDRATE METABOLISM.

XVI. THE RELATION OF EPINEPHRINE GLYCOSURIA TO DOSAGE AND TO THE CHARACTER OF THE DIET.

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(Received for publication, January 18, 1917.)

Recently it was observed by Underhill (1916) that the glycosuria (and hyperglycemia) produced by subcutaneous injections of epinephrine is decreased by the intravenous injection of alkali (sodium carbonate, in 0.5 per cent solution). Here Underhill used doses of 1 mg. of epinephrine (Parke, Davis and Company, adrenalin chloride, 1: 1,000) per kilo of body weight. In planning to extend this work, it was considered possible that the effect of the alkali in reducing the glycosuria and hyperglycemia might be more marked if a minimum effective dose of epinephrine were administered. Accordingly, the relation of glycosuria to variations in the quantity of epinephrine administered has been determined. The influence of acid-forming and base-forming diets upon the glycosuria brought about by given doses of epinephrine has also been investigated.

Methods.

Eighteen full-grown rabbits were divided into three groups, A, B, and C, each group being fed for 1 week with (1) a mixed diet of oats, cracked corn, bread, carrots, celery, grass; (2) a base-forming diet of carrots and celery; (3) an acid-forming diet of oats and cracked corn; (4) a mixed diet similar to that of the first week. A surplus of the mixed and grain diets was always available, while the base-producing food was usually entirely consumed. An abundance of water was supplied. Epinephrine (adrenalin chloride, 1: 1,000, Parke, Davis and Company) was administered in doses of 0.1 to 0.5 mg. per kilo of body weight.

TABLE I.

The Relation of Epinephrine Glycosuria to Dosage and to the Character of the Diet. Group A.

Rabbit.	32	33	34	35	36	29	50
Epinephrine per kilo, mg.	0.5	0.4	0.3	0.2	0.3	0.2	0.2
May 15–16. Base-forming diet.							
Weight, gm.	1,460	2,040	1,700	1,940	1,500	2,140	
Urine volume, cc.	175	270	115	70	230	210	
Glucose, gm.	0.163	0.0763	0.0		0.01	0.023	
May 23–24. Mixed diet.							
Weight, gm.	1,540	2,100	1,660	2,120	1,520	2,260	
Urine volume, cc.	130	170	155	155	75	145	
Glucose, gm.	0.663	0.097	0.112	0.184	1.424	0.398	
May 29–30. Acid-forming diet.							
Weight, gm.	1,520		1,580	1,900	1,540	2,080	1,820*
Urine volume, cc.	100		50	50	60	80	105
Glucose, gm.	0.139		0.042	0.065	0.559	0.278	0.017
June 5–6. Mixed diet.							
Weight, gm.	1,480		1,700	2,160	1,520	2,040	1,800
Urine volume, cc.	125		240	170	55	160	105
Glucose, gm.	0.476		0.000	0.101	0.767	1.015	0.012

* Mixed.

TABLE II.

The Relation of Epinephrine Glycosuria to Dosage and to the Character of the Diet. Group B.

Rabbit.	37	38	39	40	41	42	51
Epinephrine per kilo, mg.	0.4	0.4	0.3	0.3	0.2	0.2	0.2
May 17–18. Mixed diet.							
Weight, gm.	1,460	2,060	1,380	2,440	1,600	1,400	
Urine volume, cc.	105	150	190	325	90	60	
Glucose, gm.	1.887	1.807	0.103	3.456	0.289	0.562	
May 25–26. Base-forming diet.							
Weight, gm.	1,540	2,160	1,420	2,600	1,460	1,220	
Urine volume, cc.	205	325	240	350	210	230	
Glucose, gm.	0.807	1.764	0.789	2.079	0.397	0.065	
June 1–2. Acid-forming diet.							
Weight, gm.	1,540	2,020	1,400	2,060	1,460		1,780*
Urine volume, cc.	80	80	15	75	55		75
Glucose, gm.	1.076	0.724	0.344	0.333	0.055		0.026
June 8–9. Mixed diet.							
Weight, gm.	1,540	2,160	1,460	2,460	1,620		1,700
Urine volume, cc.	140	95	85	225	105		60
Glucose, gm.	1.77	1.045	1.120	3.915	0.134		0.65

* Mixed.

At the end of each week, each rabbit was given subcutaneously the specified dose of epinephrine and placed in a metabolism cage for 24 hours. Sugar in the urine was determined gravimetrically according to Allihn's method, the copper being weighed as cupric oxide.

TABLE III.

The Relation of Epinephrine Glycosuria to Dosage and to the Character of the Diet. Group C.

Rabbit.	43	44	45	46	47	48	49
Epinephrine per kilo, mg.	0.3	0.3	0.2	0.2	0.1	0.1	0.1
May 19–20. Mixed diet.							
Weight, gm.....	1,640	2,720	2,660	1,680	1,880	2,500	
Urine volume, cc.....	75	175	155	50	140	160	
Glucose, gm.....	0.015	4.518	1.697	0.023	0.000	0.161	
May 26–27. Base-forming diet.							
Weight, gm.....	1,500	2,260	2,600	1,680	1,600		1,640*
Urine volume, cc.....	350	115	250	240	240		110
Glucose, gm.....	1.474	2.146	0.124	0.49	0.173		
June 2–3. Acid-forming diet.						No. 52*	
Weight, gm.....		1,980	2,600	1,760	1,740	2,140	1,420
Urine volume, cc.....		130	115	45	45	60	50
Glucose, gm.....		3.480	0.815	0.074	0.063	1.039	0.022
June 9–10. Mixed diet.						No. 53*	
Weight, gm..		2,080	2,580	1,620	1,740	2,740	1,480
Urine volume, cc		125	155	75	35	130	45
Glucose, gm.....		2.720	1.400	0.150	0.000	3.583	0.030

* Mixed.

The Relation of Glycosuria to Variations in the Quantity of Epinephrine Administered.

From Tables IV and V, arranged according to the dosage, it may be seen that 0.4 mg. of epinephrine per kilo always induced glycosuria; that 0.3 mg. per kilo caused glycosuria in every instance except two (Rabbit 34), and that the average excretion of sugar incident to this dose was greater than that following the 0.4 mg. dose, although the small number of rabbits receiving the latter amount reduces the value of this comparison. With a dose of 0.2 mg. of epinephrine per kilo, glycosuria resulted in every instance, although the average amount of sugar eliminated

was less than one-third of that excreted after a dose of 0.3 mg. per kilo. A dose of 0.1 mg. of epinephrine per kilo was given only to three animals (Rabbits 47, 48, and 49, Table III). This

TABLE IV.

The Output of Sugar in the Urine Following Varying Doses of Epinephrine, in Relation to Changes in the Character of the Diet.

Rabbit.	Epinephrine per kilo.	Glucose in urine with			
		Mixed diet.	Basic diet.	Acid diet.	Mixed diet.
	mg.	*gm.*	*gm.*	*gm.*	*gm.*
33	0.4	0.097	0.076		
37	0.4	1.887	0.807	1.076	1.77
38	0.4	1.807	1.764	0.724	1.045
Average		**1.264**	**0.882**	**0.900**	**1.407**
34	0.3	0.112	0.000	0.042	0.000
36	0.3	1.424	0.01	0.559	0.767
39	0.3	0.103	0.789	0.344	1.120
40	0.3	3.456	2.079	0.333	3.915
43	0.3	0.015	1.474		
44	0.3	4.518	2.146	3.480	2.720
52	0.3				1.039
53	0.3				3.583
Average		**1.605**	**1.083**	**0.952**	**1.878**
35	0.2	0.184		0.065	0.101
29	0.2	0.398	0.023	0.278	1.015
41	0.2	0.289	0.397	0.055	0.134
42	0.2	0.562	0.065		
45	0.2	1.697	0.124	0.815	1.400
46	0.2	0.023	0.49	0.074	0.150
50	0.2				0.017
50	0.2				0.012
51	0.2				0.026
51	0.2				0.650
Average		**0.526**	**0.22**	**0.257**	**0.389**
General average		1.105	0.732	0.654	1.081

amount failed to produce glycosuria in two cases out of seven. In view of these results it seems probable that a dose of 0.3 mg. of epinephrine per kilo of body weight may be relied upon to induce glycosuria when the liver contains a sufficiency of glycogen.

TABLE V.

The Output of Sugar in the Urine Following Varying Doses of Epinephrine, in Relation to Changes in the Character of the Diet. Results for Animals That Survived the Entire Experimental Period.

Rabbit.	Epinephrine per kilo.	Glucose in urine with			
		Mixed diet.	Basic diet.	Acid diet.	Mixed diet.
	mg.	*gm.*	*gm.*	*gm.*	*gm.*
37	0.4	1.887	0.807	1.076	1.77
38	0.4	1.807	1.764	0.724	1.045
Average.....	.....	**1.847**	**1.285**	**0.900**	**1.407**
34	0.3	0.112	0.000	0.042	0.000
36	0.3	1.424	0.01	0.559	0.767
39	0.3	0.103	0.789	0.344	1.120
40	0.3	3.456	2.079	0.333	3.915
44	0.3	4.518	2.146	3.480	2.720
Average.....	.....	**1.923**	**1.005**	**0.952**	**1.704**
35	0.2	0.184		0.065	0.101
29	0.2	0.398	0.023	0.278	1.015
41	0.2	0.289	0.397	0.055	0.134
45	0.2	1.697	0.124	0.815	1.400
46	0.2	0.023	0.49	0.074	0.150
Average.....	.....	**0.518**	**0.258**	**0.257**	**0.560**
General average..		1.325	0.784	0.654	1.178

The Influence of Changes in the Character of the Diet upon Epinephrine Glycosuria.

The influence of the character of the diet is fairly uniform (see Tables IV and V). The output of sugar was greatest when the rabbits were given a mixed diet, and there is good agreement between the results of repeated doses upon this diet. The acid-forming diet resulted in the lowest output of sugar, averaging slightly more than one-half that upon a mixed diet. During the base-forming diet the sugar excretion was greater than with that yielding an acid ash, although the excess was, in general, slight—not more than 20 per cent.

Inasmuch as the glycosuria produced by epinephrine is dependent upon the animal's store of glycogen, these results apparently indicate that an acid-forming diet is slightly less effi-

cient in the formation of glycogen than is a base-forming diet, and that both are greatly inferior to a mixed diet in this respect. The variations in body weight seem to point in the same direction, gain in weight having been greatest and most frequent upon the mixed diet. In a comparison of the acid- and base-producing diets, gain in weight is more often seen with that yielding a basic ash.

According to Allen, the excretion of sugar after epinephrine administration is markedly influenced by diuresis. In these experiments the volume of urine was much less during the acid-forming diet of grain than with the base-producing diet of fresh vegetables, owing to the small amount of water taken by the animals while upon the grain diet. This fact may be partly responsible for the reduced output of sugar following the acid-forming diet, although an examination of the figures indicates no constant relation between the volume of urine and its sugar content.

CONCLUSIONS.

A dose of 0.3 mg. of epinephrine per kilo of body weight may be depended upon to produce glycosuria in a normal rabbit.

With a sufficient quantity of food, rabbits upon a mixed diet excrete larger amounts of sugar after epinephrine administration than when maintained upon either an acid-producing diet or one yielding a basic ash. The data given indicate that during a base-forming diet a somewhat larger output of sugar occurs subsequent to epinephrine injection than obtains under similar conditions with an acid-producing diet.

These results may possibly be interpreted to mean that there is a greater glycogen storage upon a base-producing diet than upon a dietary yielding an acid ash.

With injections of epinephrine repeated at intervals of 1 week, with rabbits there is no apparent decreased susceptibility to the drug.

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STUDIES IN CARBOHYDRATE METABOLISM.

XVII. FURTHER EXPERIMENTS UPON THE INFLUENCE OF THE INTRAVENOUS INJECTION OF SODIUM CARBONATE UPON EPINEPHRINE HYPERGLYCEMIA AND GLYCOSURIA.

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(Received for publication, January 18, 1917.)

In previous papers it has been shown that carbohydrate metabolism, in normal animals, is usually undisturbed by injections of sodium carbonate or by diets yielding an acid or basic ash. When, however, the sugar-regulating mechanism is thrown out of equilibrium, pronounced effects may result from the introduction of acid or alkali.[1]

Recently Underhill demonstrated that the hyperglycemia and glycosuria due to epinephrine are decreased by the administration of sodium carbonate. In the experiments reported in the present paper, this investigation has been extended with the object of determining whether, with the minimum effective dose of epinephrine, 0.3 mg. per kilo of body weight,[2] hyperglycemia and glycosuria can be entirely prevented by the injection of sodium carbonate.

Methods.

The experiments were performed upon healthy, full-grown rabbits. Injections into the ear vein of a solution of sodium carbonate (0.5 per cent) were followed in 30 minutes by subcutaneous administrations of 0.3 mg. of epinephrine (Parke, Davis and Company, adrenalin chloride, 1: 1,000) per kilo of body weight. Samples of blood for the determination of sugar were

[1] McDanell, L., and Underhill, F. P., *J. Biol. Chem.*, 1917, xxix, 227.

[2] McDanell and Underhill, *J. Biol. Chem.*, 1917, xxix, 245.

taken before the administration of the carbonate, ½ hour afterward (just preceding the injection of epinephrine), and subsequently at intervals of 1 hour. The method of Lewis and Benedict was used for the estimation of blood sugar. The sugar in the urine was determined by the Allihn procedure, the copper being weighed as cupric oxide.

The data obtained may be found in Tables I and II, and in Fig. 1 the results are expressed in the form of curves. In Table I (last line) the averages for sugar of the blood and urine obtained

TABLE I.

Hyperglycemia and Glycosuria Resulting from Injections of Epinephrine. 0.3 Mg. per Kilo.

Date.	Rabbit.	Body weight.	Blood sugar content (in percentages).								Urine.	
			Normal.	Hrs. after injection of epinephrine.							Volume.	Sugar content.
				1	2	3	4	5	6	7		
		gm.									*cc.*	*gm.*
June 13	29	2,100	0.113	0.223	0.19	0.16	0.131	0.115	0.099	0.10	80	0.480
" 13	36	1,560	0.113	0.294	0.30	0.285	0.232	0.205	0.144	0.117	175	1.855
" 16	50	1,920	0.132	0.304	0.30	0.273	0.187	0.128	0.118	0.106	225	2.358
" 16	38	1,940	0.114	0.302	0.256	0.194	0.156	0.116	0.109	0.114	175	0.342
Averages.....			**0.118**	**0.281**	**0.261**	**0.228**	**0.176**	**0.141**	**0.117**	**0.109**		**1.259**
(Underhill).....			0.13	0.36	0.430	0.46		0.37	0.32	0.19*		1.49

* After 7½ hours.

by Underhill with a dose of 1 mg. of epinephrine per kilo are included for comparison. It is evident that the hyperglycemia incident to injections of the larger dose of epinephrine is considerably greater and of longer duration than that which follows the administration of 0.3 mg. of epinephrine per kilo. Also, the maximun blood sugar content is reached sooner in the latter instance, the normal being regained in about 6 hours, or less. The average output of sugar in the urine is, however, only about 15 per cent less with the small dose of epinephrine than with injections more than three times as large.

The injection of approximately 0.5 gm. of sodium carbonate in 0.5 per cent solution ½ hour previous to the administration of epinephrine brings about a considerable diminution in the hyper-

TABLE II.

The Influence of the Intravenous Injection of Sodium Carbonate (0.5 per Cent) upon Epinephrine Hyperglycemia and Glycosuria.

Date	Rabbit.	Body weight.	Volume of sodium carbonate solution injected.	Blood sugar content (in percentages).								Urine.	
					Hrs. after injection of								
					Sodium carbonate.	Epinephrine.							
				Normal.	½	1	2	3	4	5	6	Volume.	Sugar content.
		gm.	cc.									cc.	gm.
June 19	40	2,560	120	0.10	0.112	0.323	0.237	0.153	0.127	0.112	0.095	285	0.464
" 19	39	1,540	100	0.115	0.109	0.257	0.251	0.16	0.149	0.141	0.127	200	0.524
" 22	44	2,120	125	0.111	0.131	0.297	0.350	0.344	0.295	0.289	0.228	245	2.773
" 22	49	1,620	100	0.117	0.074	0.255	0.172	0.155	0.132	0.134	0.104	250	0.147
" 26	45	2,560	125	0.111	0.093	0.167	0.168	0.128	0.117	0.108	0.093	275	0.000
" 26	53	2,640	125	0.115	0.121	0.216	0.254	0.247	0.172	0.141	0.119	220	0.000
" 28	38	1,800	110	0.106	0.096	0.224	0.217	0.185	0.146	0.129	0.108	215	0.000
" 28	50	1,800	110	0.111	0.108	0.206	0.210	0.201	0.176	0.167	0.124	230	0.156
Averages.....				**0.111**	**0.105**	**0.243**	**0.232**	**0.197**	**0.164**	**0.153**	**0.124**		**0.509**

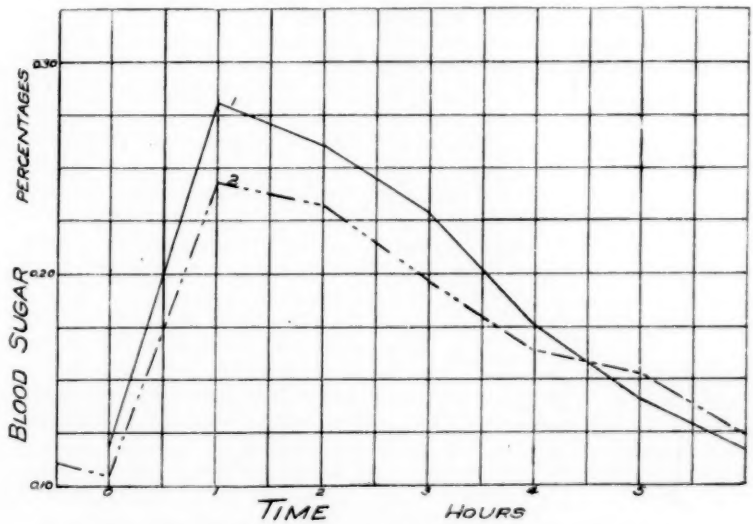

FIG. 1. Curves showing the blood sugar level after subcutaneous injections of epinephrine and the influence of the intravenous administration of sodium carbonate upon the hyperglycemia induced (average figures).

Curve 1. Epinephrine only.

Curve 2. Epinephrine and sodium carbonate.

glycemia caused by epinephrine (see Table II), although the reduction is proportionately less than that obtained by Underhill following the larger dose of epinephrine and, in some instances, larger injections of sodium carbonate. Accompanying the diminished hyperglycemia is a noticeable reduction in the output of sugar in the urine.

CONCLUSIONS.

The present experiments confirm the statement of Underhill: "The hyperglycemia and glycosuria provoked by epinephrine are both significantly decreased if sodium carbonate is administered at suitable periods of time previous to epinephrine introduction."

Intravenous injection of sodium carbonate modifies significantly the influence of the minimum effective dose of epinephrine, but does not prevent the occurrence of hyperglycemia and glycosuria. In fact, it appears that, with the smaller dose of epinephrine, hyperglycemia and glycosuria are diminished relatively less by sodium carbonate than when larger doses of epinephrine are administered.

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STUDIES IN CARBOHYDRATE METABOLISM.

XVIII. THE RELATION OF DIET TO THE GLYCOGEN CONTENT OF THE LIVER.

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It has been accepted for a long time that glycosuria accompanies partial asphyxiation. Alvaro Reynoso (see Dastre) appears to have been the first to notice this, but he made no further accurate observations on the subject. Claude Bernard confirmed this observation and found also that prolonged asphyxia caused the disappearance of glycogen from the liver. This work of Bernard was extended by Dastre, who noted that the percentage of reducing substance in the blood rose from 0.128 per cent when a dog was breathing air, to 0.253 per cent when breathing was continued for some time in a confined space. The hyperglycemia was ascribed to lack of oxygen in the blood. Araki in 1891 reported that glycosuria was caused in dogs, rabbits, and chickens by insufficient oxygen supply, although no analyses of the air breathed or of the blood gases were given.

This idea seems to have represented the predominant view of observers until Edie, as the result of experiments in which the composition of the air breathed was determined, reached the conclusion that the glycosuria occurring after partial asphyxiation is due to excess of carbon dioxide, rather than to lack of oxygen. He found that a low percentage of oxygen alone—less than 6 per cent—did not produce glycosuria, while it resulted from breathing air containing 10 to 15 per cent by volume of carbon dioxide, even if the percentage of oxygen was greater than that normally present in the atmosphere. Edie placed carbon dioxide in the class with anesthetics, all of which cause glycosuria.

This work was confirmed and extended by Edie, Moore, and Roaf. They concluded that the glycosuria attributed by Araki to low oxygen supply was actually due to accumulation of carbon dioxide. Araki's method of freeing the air from carbon dioxide—bubbling it through strong caustic potash—they found, by gas analysis, to be totally inadequate for the purpose.

In agreement with these findings of Edie and his coworkers is the conclusion of Macleod, that the property of asphyxial blood which stimulates hepatic glycogenolysis in the intact animal is the excess of carbon dioxide, and not the deficiency of oxygen which it contains.

On the other hand, that certain types of glycosuria are accompanied by a marked acapnia, rather than by an excess of carbon dioxide, was shown by Henderson and Underhill. They made analyses of the blood gases following peptone injection, piqûre, removal of the pancreas, painting of the pancreas with piperidine, laparotomy, and excessive artificial respiration. In all of these conditions they found acapnia. By supplying carbon dioxide, these investigators were able, in some instances, to prevent disturbances of the sugar-regulating mechanism.

Pavy and Bywaters, in a study of the influence of environment on enzyme action, demonstrated that with invertin (yeast) and diastase (oyster) the activity of the enzyme is increased by the addition of small quantities of acetic acid until an optimum point is reached, after which the addition of more acid leads to a diminution in the enzymic power. They showed, further, that the activity given by acid addition is susceptible of removal by a basic substance,—ammonia, sodium carbonate, and even the basic material of tap water exerting an inhibiting influence, which the subsequent addition of an acid is capable of counteracting. An environment of strong acid or alkali destroys the enzyme. With invertin, it was observed that carbon dioxide approximates acetic acid in its effect in promoting enzyme action, and with oyster diastase, the effect is similar although much reduced.

In the case of higher animals these investigators found that the enzymic power of the liver, post mortem, in which a fully activated enzyme exists, is usually not increased by addition of acid, but rather is diminished. They determined, too, that the postmortem production of sugar in the liver may be virtually checked by the injection of a 2 per cent solution of sodium carbonate into the portal system of the living animal. The addition of increasing quantities of acetic acid to the liver thus treated brings about an augmentation of enzyme activity until an optimum is reached, after which the further addition of acid leads to a decline of power. The postmortem production of acid in the liver is similar to that of sugar, of which, according to these authors, it may be the precursor. A restraining influence is exerted by a fluoride upon postmortem acid production in the liver as is done with postmortem sugar formation.

Later an attempt was made by Pavy and Godden to determine if the production of sugar by the liver, occurring in association with glycosuria, could be similarly checked, and the glycosuria thus removed, by the injection of sodium carbonate into the circulation. Chloroform glycosuria, being dependent upon the glycogen supply of the liver, was used for testing the effect of the sodium carbonate. A cat was placed under the influence of chloroform, and urine withdrawn at 15 minute intervals by means of a catheter. After glycosuria had been thoroughly established, sodium carbonate, in 3 per cent solution, to the amount of 0.75 gm. per kilo of body weight, was injected into the femoral vein. This brought about a speedy reduction in the previously existing glycosuria, which usually disappeared within 15 to 30 minutes after the completion of the injection. When normal saline or Ringer's solution was substituted for the sodium

carbonate, it exerted no influence upon the glycosuria. They found further, that, after the injection of the sodium carbonate and the consequent reduction of the glycosuria, pinching the vagus produced no effect, showing that the nerve stimulation of glycosuria was, like the chloroform glycosuria, held in check by the agency of the carbonate. In conclusion, these investigators say: "Seeing that there is evidence to show that both forms of glycosuria are due to an abnormal enzymic conversion of glycogen into sugar (attributable, there are grounds for suggesting, to acidosis development) there is nothing unconformable with what may be reasonably looked for."

Macleod and Pearce noted that after death in an etherized animal, there is usually, but not always, an acceleration in the rate of glycogenolysis.

As far back as 1882, Einhorn, working in Ehrlich's laboratory (see Ehrlich), showed that while winter frogs neither lose glycogen from their livers, nor form new glycogen, when immersed in plain sugar solution, they do lose their glycogen if the sugar solution is acidulated. But when such frogs were placed in a glucose-sodium-carbonate solution, a formation of new glycogen from available glucose, without the occurrence of glycogenolysis, was demonstrated.

In turtle livers, perfused with Ringer's solution containing hydrochloric acid, Elias observed a considerable loss of glycogen. When sodium carbonate was added to the Ringer's solution, he noted an actual glycogenesis. Elias also brought about a loss of glycogen from the livers of rabbits by the administration of 0.25 N hydrochloric acid.

As the result of a limited number of experiments, Kramer, Marker, and Murlin suggested tentatively that the glucose retained as a result of the administration of sodium carbonate to a depancreatized dog is not held back as glycogen.

While the results cited indicate that, in normal animals and in those with certain disturbances of the sugar-regulating mechanism, a large excess of alkali contributes to the formation of glycogen, and acid increases the activity of the glycogenolytic process, the influence of an acid-forming or base-forming diet upon the glycogen content of the liver seems not to have been investigated. Experiments conducted for this purpose are described in the present paper. The investigation is especially pertinent in view of the results reported in a preceding paper,[1] where there were indications that the character of the diet (its acid-base-producing properties) plays a rôle in the degree of glycosuria induced by epinephrine administration.

[1] McDanell, L., and Underhill, F. P., *J. Biol. Chem.*, 1917, xxix, 245.

Series I.

Methods.—A subcutaneous injection of epinephrine (Parke, Davis and Company, adrenalin chloride, 1: 1,000), 1 mg. per kilo of body weight, was given to each of ten full-grown rabbits for the purpose of freeing their livers of the excess of glycogen. The rabbits, in two groups of five each, were placed in pens. To those of Group A was given a base-producing diet of carrots, about a pound daily for each rabbit. Group B received an ample acid-producing diet of oats and cracked corn. Water was always available. One rabbit of Group B died 4 days later and was not replaced. The animals were kept upon the designated rations

TABLE I.

The Influence of Diet upon the Glycogen Content of the Liver.
Series I, Group A, July 11–17, Carrot Diet.

Rabbit	29	34	36	38	40	Average.
Weight						
July 11, gm.	2,240	1,700	1,540	1,720	2,520	
" 17, "	2,200	1,700	1,440	1,720	2,500	
Weight of liver, gm.	52	53	42	49	82	
Glycogen.						
Glucose × 0.927, gm.	2.83	3.65	1.86	2.08	3.12	2.71
Per cent of weight of liver.	5.44	6.91	4.43	4.24	3.81	4.87
Per kilo of body weight, gm.	1.28	2.15	1.29	1.21	1.25	1.42

for 6 days, then killed, and the glycogen in the livers was estimated according to Pflüger's method.[2] After hydrolysis the glucose was determined by the Allihn procedure, the copper being weighed as cupric oxide.

From Table I it may be seen that, of the rabbits in Group A upon the carrot diet, two maintained their weight, while one lost 20, another 40, and another 100 gm. However, the percentage of glycogen in the livers and the amount of glycogen per kilo of body weight have no direct relation to these slight changes in weight. It is evident that the variation in amount of

[2] Pflüger, E., *Arch. ges. Physiol.*, 1906, cxiv, 242; *Abderhalden's Handb. biochem. Arbeitsmethoden*, 1910, ii, 162.

glycogen per kilo of body weight, with the exception of Rabbit 34, is slight.

Table II shows that the rabbits of Group B, upon an unlimited grain diet, all lost weight, from 20 to 300 gm.; and that, with the exception of Rabbit 53, the percentage of glycogen in the livers, as well as the amount per kilo of body weight, was less than that of the rabbits of Group A, upon the carrot diet. However, the fact that Rabbit 53 stored a much greater proportion of glycogen than did any of the rabbits of Group A indicated that there were undoubtedly other factors than the character of the diet to be considered. Since this rabbit had practically maintained its weight, while the others had lost considerably, it seemed possible

TABLE II.

The Influence of Diet upon the Glycogen Content of the Liver.

Series I, Group B, July 11–17, Grain Diet.

Rabbit.	45	50	51	53	Average
Weight.					
July 11, gm.	2,480	1,740	1,860	2,500	
" 17, "	2,300	1,440	1,780	2,480	
Weight of liver, gm.	60	46	47	70.5	
Glycogen.					
Glucose × 0.927, gm.	0.697	1.055	1.693	6.956	2.6
Per cent of weight of liver.	1.16	2.29	3.6	9.87	4.65
Per kilo of body weight, gm.	0.303	0.733	0.951	2.805	1.3

that they had failed to store glycogen because of an insufficient intake of food, as it is our experience that many rabbits do not relish a diet of grain only.

While these results apparently indicated a better storage of glycogen upon a base-forming than upon an acid-forming diet, further experiments were planned in which the amount of food consumed by each rabbit should be known.

Series II.

Methods.—The rabbits of Series II were kept in separate metabolism cages for 1 week. After the preliminary injection of epinephrine, those of Group A were given 500 gm. of carrots a day;

those of Group B, 100 gm. of oats (instead of corn and oats). One rabbit of Group A died on the 3rd day. The food value of the diets was estimated from the percentage composition of carrots given by Sherman;[3] and of oats, by Henry and Morrison.[4]

TABLE III.

The Influence of Diet upon the Glycogen Content of the Liver. Series II, Group A, October 23–30, Carrot Diet.

Rabbit.	73	76	77	Average.
Weight.				
October 23, gm.	2,520	1,920	1,900	
" 30, "	2,740	1,920	1,920	
Food eaten.				
October 24, gm.	98	500	480	
" 25, "	327	500	467	
" 26, "	500	500	380	
" 27, "	500	500	500	
" 28, "	500	500	500	
" 29, "	500	500	500	
" 30, "	470	500	366	
Total, gm.	2,895	3,500	3,193	
Carbohydrate, gm.	214	259	236	
Per kilo, gm.	78.09	134.9	122.9	111.96
Calories	1,038	1,256	1,145	
Per kilo	378.7	654.2	596.4	543.1
Weight of liver, gm.	81.8	70.7	69.8	
Glycogen.				
Glucose × 0.927, gm.	7.1	5.16	4.94	5.73
Per cent of weight of liver	8.67	7.3	7.08	7.68
Per kilo of body weight, gm.	2.59	2.69	2.57	2.62
Per 100 gm. carbohydrate, gm.	3.24	1.99	2.09	2.44
Per 1,000 calories, gm.	6.84	4.11	4.31	5.09

Table III shows that in Series II none of the rabbits of Group A upon the carrot diet lost weight, one of them (Rabbit 73) gaining 220 gm.; and that they all stored more glycogen, absolutely and relatively, than the carrot-fed rabbits of Series I (see Table I).

[3] Sherman, H. C., The Chemistry of Food and Nutrition, New York, 1911, 323.

[4] Henry, W. A., and Morrison, F. B., Feeds and Feeding, New York, 1915, 634.

That great variation was manifest in Group B of Series II, as of Series I, is indicated in Table IV. Two of the rabbits gained weight (Rabbits 68 and 71, 20 and 100 gm. respectively), while

TABLE IV.

The Influence of Diet upon the Glycogen Content of the Liver.

Series II, Group B, October 23–30, Oat Diet.

Rabbit.	68	69	70	71	72	75	Average.
Weight.							
October 23, gm..	2,360	2,520	2,760	1,720	2,580	2,600	
" 30, " ..	2,380	2,460	2,560	1,820	2,500	2,440	
Food eaten.							
October 24, gm..	29	10	39	36	25	7	
" 25, " ..	85	22	17	83	94	70	
" 26, " ..	95	42	65	90	98	75	
" 27, " ..	95	68	91	78	98	93	
" 28, " ..	50	66	72	43	98	90	
" 29, " ..	95	65	80	100	100	50	
" 30, " ..	88	67	49	65	92	19	
Total, gm.....	537	340	413	495	605	404	
Carbohydrate, gm.....	320	203	246	295	361	241	
Per kilo, gm.	134.5	82.52	96.1	162.1	144.4	98.77	119.73
Calories.....	1,805	1,144	1,318	1,664	2,035	1,359	
Per kilo.....	758.4	465	514.8	914.3	814.1	556.9	670.6
Weight of liver, gm.....	79.5	67.5	75.6	65.1	102.9	68.4	
Glycogen.							
Glucose × 0.927, gm.....	8.68	4.43	3.05	5.97	11.61	0.92	5.78
Per cent of weight of liver.....	10.91	6.56	4.04	9.18	11.29	1.34	7.22
Per kilo of body weight, gm....	3.65	1.8	1.19	3.28	4.64	0.38	2.49
Per 100 gm. carbohydrate, gm.	2.71	2.18	1.24	2.03	3.22	0.38	1.96
Per 1,000 calories, gm.....	4.81	3.87	2.31	3.59	5.7	0.68	3.49

the other four lost, the amount varying from 60 to 200 gm. Rabbits 68 and 72 stored more glycogen, and Rabbits 68, 71, and 72 each showed a greater percentage of glycogen in the liver, as well as a

larger amount of glycogen per kilo of body weight, than did any of the carrot-fed rabbits. Much less glycogen was stored by the other three rabbits of this group than by those of Group A. When averages are considered, it may be seen that the proportion of glycogen in the liver and the amount per kilo of body weight were slightly greater with the carrot-fed than with the oat-fed animals, irrespective of the food intake.

The importance of the quantity of food ingested is, however, demonstrated by the results in Series II. Rabbits 68, 71, and 72 (Table IV), which stored the greatest proportion of glycogen, received by far the largest number of calories per kilo. Rabbit 75, which stored the least glycogen, ate less during the last 2 days than any of the others, although its total food intake was not the lowest.

It is equally evident from a study of Tables III and IV that the quantity of food consumed is not the sole determinant of the glycogen storage. Of the six rabbits in Group B, oat-fed (Table IV), four (Rabbits 69, 70, 71, 75) stored less glycogen per 1,000 calories of food eaten than any of the rabbits of Group A, carrot-fed (Table III), only one of Group B (Rabbit 72) manifesting greater ability in this respect than the average of Group A. This oat-fed rabbit (No. 72), which stored 11.6 gm. of glycogen, received more than twice as many calories per kilo as Rabbit 73, carrot-fed, which stored 7.1 gm. of glycogen. In fact, the 7.1 gm. of glycogen were stored by Rabbit 73, upon a base-forming diet, with an intake of 378.7 calories per kilo. The lowest fuel value of the diet of any of the oat-fed rabbits was 465 calories per kilo (Rabbit 69). Although the average fuel value of the acid-forming diet of the rabbits of Group B (670.6 calories) was more than 20 per cent greater than that of the base-forming diet of Group A (543.1 calories), the average amount of glycogen stored per 1,000 calories was 30 per cent less in the former instance than in the latter. With respect to the efficiency of the carbohydrate in the two diets, the differences, although less, point in the same direction. With an average intake of carbohydrate per kilo slightly greater, the rabbits upon an acid-forming diet (Group B) stored about 20 per cent less glycogen per 100 gm. of carbohydrate received than those upon the base-producing diet (Group A).

CONCLUSIONS.

The results obtained indicate that in normal rabbits a base-forming diet is somewhat more efficient in the formation of glycogen than an acid-producing diet. This is in agreement with the results of previous investigators, who have found that an excess of alkali contributes to the accumulation of glycogen. Notwithstanding this fact, it has also been demonstrated that a large storage of glycogen may take place upon an acid-forming diet when sufficient food is ingested.

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STUDIES IN CARBOHYDRATE METABOLISM.

XIX. THE INFLUENCE OF THE INTRAVENOUS INJECTION OF SODIUM CARBONATE UPON THE HYPERGLYCEMIA AND GLYCOSURIA FOLLOWING THE SUBCUTANEOUS ADMINISTRATION OF GLUCOSE.

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In the normal organism of the rabbit, the blood-sugar-regulating mechanism is usually undisturbed by injections of alkali or by the ingestion of an acid- or base-producing diet. On the other hand, there is evidence that the glycogenetic function of the body is more favorably influenced by a base-forming diet than it is by a dietary yielding an acid ash.[1] Under conditions where the sugar-regulating mechanism is thrown out of equilibrium, as by epinephrine administration, the introduction of alkali exerts a distinct influence upon carbohydrate metabolism, as evidenced by the diminished output of sugar in the urine. In general, it would appear that alkali has a distinct tendency to conserve the carbohydrate of the body, and in this action the glycogenetic and glycogenolytic processes are probably actively concerned.

If the alkali shows a distinctly favorable influence upon glycogen storage, it would appear reasonable to assume that the simultaneous introduction of alkali and glucose should yield less urinary sugar than glucose alone. The present communication gives the results of an endeavor to determine this question, the problem being investigated with respect to the content of blood sugar, and the urinary sugar excretion.

[1] McDanell, L., and Underhill, F. P., *J. Biol. Chem.*, 1917, xxix, 255.

Methods.

Healthy, full-grown rabbits, upon a mixed diet, were employed for these experiments. During the first part of the work, Merck's purified dextrose was used, with the exception of three injections of commercial glucose. Afterward Kahlbaum's pure dextrose was administered. Kahlbaum's anhydrous sodium carbonate and sodium chloride were employed. After a sample of blood was obtained for the determination of the normal sugar content, the rabbit received a subcutaneous injection of glucose, about 5 gm. per kilo of body weight, in 30 per cent solution. The blood sugar content was determined by the Lewis-Benedict method at the end of ½ hour and 1 hour, and each subsequent hour until the 5th. When sodium carbonate was given, an infusion of approximately 0.5 gm., in 0.5 per cent solution, was made into the ear vein 15 minutes after the glucose injection. In substituting sodium chloride for the carbonate, a 0.364 per cent solution was employed, that being isotonic with the 0.5 per cent sodium carbonate.[2] To some animals three or four injections were given. Sugar was injected at intervals of not less than 1 week. The animals were kept in metabolism cages and the urine was collected for 24 hours after each injection. Urinary sugar was estimated according to the Allihn procedure.

The Hyperglycemia and Glycosuria Following the Subcutaneous Administration of Glucose.

According to Allen, the rabbit possesses a greater assimilation limit for glucose than the dog. This assertion is based upon the work of various investigators, particularly Heilner and Fichtenmayer. A perusal of the literature emphasizes, however, that there is a diversity of opinion concerning the assimilation limit of the rabbit for subcutaneously injected glucose. Thus, Frugoni and Stradiotti place the threshold dose for the rabbit at 2.35 gm. per kilo, as compared with 3 gm. per kilo for the guinea pig. Allen designates these results atypical. Süssenguth injected 5 to 10 gm. of glucose per kilo, and observed glycosuria with the

[2] Jones, H. C., *Z. physik. Chem.*, 1893, xi, 113; 1893, xii, 636. Abegg, R., Handb. anorgan. Chem., Leipsic, 1905, ii, pt. i, 228, 302.

larger doses. Marrassini reported glycosuria usually after doses of approximately 10 gm. of glucose per kilo. Heilner, with doses of 12 to 13 gm. of glucose per kilo, observed slight glycosuria in some instances, and none in others. One rabbit showed neither glucose nor albumin in the urine after a subcutaneous dose of 49.8 gm. of glucose. Fichtenmayer reported no glycosuria in rabbits, some of them fasting, with injections of glucose in amounts varying from 10 to 23 gm. per kilo. Allen observed

TABLE I.

The Hyperglycemia and Glycosuria Resulting from the Subcutaneous Injection of a 30 per Cent Solution of Glucose.

First Injections.

Date.	Rabbit.	Weight.	Volume of glucose solution injected.	Blood sugar content (in percentages).							Urine.	
				Normal.	Hrs. after injection of glucose.						Volume	Sugar content.
					½	1	2	3	4	5		
		gm.	*cc.*								*cc.*	*gm.*
July 11..	54	2,620	44*	0.134	0.273	0.315	0.287	0.287	0.21	0.18	70	0.86
" 11..	55	3,040	50.5*	0.107	0.215	0.205	0.169	0.186	0.153	0.155	43	0.39
" 13..	57	2,460	41*	0.105	0.226	0.239	0.258	0.226	0.183	0.145	120	0.516
" 13..	58	2,720	44*	0.116	0.217	0.235	0.238	0.20	0.175	0.171	76	0.481
" 18..	59	2,460	41*	0.091	0.256	0.282	0.271	0.229	0.177	0.146	45	0.317
" 18..	60	1,880	31.3*	0.13	0.227	0.237	0.188	0.157	0.137	0.138	23	0.271
Sept. 29..	61	2,960	44.4†	0.12	0.325	0.343	0.24	0.179	0.155	0.145	75	0.297
" 29..	62	2,260	34†	0.126	0.274	0.32	0.287	0.255	0.207	0.199	65	0.248
Oct. 2..	63	1,880	28†	0.131	0.268	0.246	0.202	0.189	0.148	0.142	49	0.035
" 2..	64	2,060	32.5†	0.12	0.238	0.24	0.173	0.114	0.131	0.136	46	0.037
" 6..	67	1,840	27.6†	0.114	0.202	0.20	0.17	0.156	0.15	0.137	28	0.081
Average.....				**0.1176**	**0.247**	**0.262**	**0.226**	**0.198**	**0.166**	**0.154**		**0.321**

* Dextrose, Merck.

† Dextrose, Kahlbaum.

glycosuria in one rabbit with a dose of 5 gm. per kilo, while others failed to develop glycosuria with doses as large as 7.7 gm. per kilo.

It is apparent from these diverse results that renewed investigation was essential for the determination of the assimilation limit for subcutaneously introduced glucose in the rabbit. It was also important to discover the influence upon the assimilation limit of repeated injections of sugar.

Our experiments have been conducted with doses of glucose approximating 5 gm. per kilo of body weight. The results may be seen in Tables I and II. Table I includes first injections, and Table II, second, third, and fourth injections. While wide variations are manifest in the assimilation limit of different animals, and of the same animal at different times, in only one instance did a dose of 4 to 5 gm. of glucose per kilo fail to produce

TABLE II.

The Hyperglycemia and Glycosuria Resulting from the Subcutaneous Injection of a 30 per Cent Solution of Glucose.

Second, Third, and Fourth Injections.

Date.	Rabbit.	Weight.	No. of injection.	Volume of glucose solution injected.	Blood sugar content (in percentages).							Urine.	
					Normal.	Hrs. after injection of glucose.						Volume.	Sugar content.
						½	1	2	3	4	5		
		gm.		*cc.*								*cc.*	*gm.*
July 31	55	2,520	4	50.5*	0.13	0.361	0.408	0.408	0.392	0.378	0.352	63	4.334
" 31	57	2,180	4	41*	0.128	0.317	0.353	0.37	0.357	0.275	0.208	65	0.796
Oct. 20	61	2,340	4	44.4†	0.102	0.231	0.24	0.192	0.178	0.181	0.216		
" 9	64	2,020	2	32.5†	0.148	0.391	0.405	0.312	0.221	0.156	0.164	45	0.296
" 16	64	1,840	3	32.5†	0.119	0.244	0.28	0.238	0.266	0.272	0.28		
" 11	65	1,900	2	27†	0.124	0.215	0.204	0.141	0.15	0.13	0.122	72	0.180
" 25	65	1,900	4	27†	0.125	0.198	0.227	0.20	0.15	0.119	0.112	110	0.000
" 11	66	1,640	2	25.2†	0.14	0.323	0.346	0.353	0.255	0.156	0.134	54	0.316
" 25	66	1,760	4	25.2†	0.144	0.281	0.292	0.304	0.26	0.160	0.122	146	0.147
" 13	67	1,740	2	27.6†	0.139	0.21	0.189	0.156	0.159	0.15	0.16	35	0.035
" 20	67	1,860	3	27.6†	0.126	0.245	0.24	0.199	0.149	0.138	0.134	84	0.115
Average					**0.129**	**0.274**	**0.289**	**0.261**	**0.231**	**0.192**	**0.182**		**0.691**

* Commercial glucose.

† Dextrose, Kahlbaum.

glycosuria (Rabbit 65, fourth injection, Table II). As indicated by the glycemia, the tolerance of this animal was above the average. According to Underhill and Closson, Underhill and Hilditch, and Allen, 5 gm. of glucose per kilo never produce glycosuria (barring accidental traces) in any normal dog. Hence the results here obtained assign to the rabbit a lower assimilation limit for subcutaneously administered glucose than is ordinarily accepted for the dog.

In general, after subcutaneous administration of glucose, the blood sugar level rises rapidly during the first half hour, and more slowly during the second half hour. A maximum is reached 1 hour after the administration of glucose, and thereafter is a gradual decline to normal.

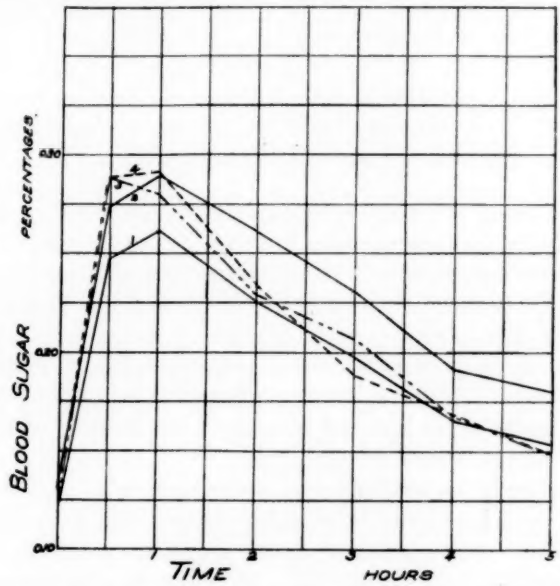

FIG. 1. Curves showing the blood sugar level after subcutaneous injections of glucose, and the influence of the intravenous administration of sodium carbonate and sodium chloride upon the hyperglycemia induced (average figures).

Curve 1. Glucose only (first injections).
" 2. " " (second, third, and fourth injections).
" 3. " and sodium carbonate.
" 4. " " " chloride.

Allen found no diminution in the power of cats and dogs to utilize subcutaneously injected glucose, as the result of repeated injections. In our experiments with rabbits, the average maximum hyperglycemia incident to first injections is just a little lower than from subsequent injections, and the average output of sugar in the urine from first injections is somewhat smaller than that obtained from the later injections. In Fig. 1 may be

seen the striking parallelism between the average curve of blood sugar content obtained from the initial sugar injection (Curve 1), and that yielded by repeated sugar administration (Curve 2). The data presented indicate that with rabbits, in general, repetition of subcutaneous injections of glucose, under the experimental conditions, is also without significant influence upon the assimilation limit of sugar.

The Influence of the Intravenous Injection of Sodium Carbonate upon the Hyperglycemia and Glycosuria Following the Subcutaneous Administration of Glucose.

Before drawing conclusions concerning the influence of sodium carbonate upon the assimilation limit of glucose, it was deemed desirable to ascertain the effect of some other solution with the same osmotic pressure, but without the presence of alkali. For this purpose the 0.364 per cent solution of sodium chloride was employed. It is evident from Table IV and Fig. 1 (Curve 4) that the sodium chloride solution is without appreciable influence upon the assimilation limit of glucose in the rabbit, under the experimental conditions selected.

When glucose is administered subcutaneously and sodium carbonate is injected into the blood stream shortly afterward, the resulting hyperglycemia shows marked variations with different animals (see Table III). If, however, an average of these figures is taken, the resulting curve which may be formed (Fig. 1, Curve 3) is similar to that yielded by the average figures for the injection of glucose alone (Curves 1 and 2, Fig. 1). The maximum is almost identical with that for repeated injections of glucose only, but is reached in $\frac{1}{2}$ hour rather than in 1 hour. The administration of sodium carbonate has no significant influence upon the glycosuria induced by the subcutaneous injection of glucose.

CONCLUSIONS.

The tolerance of the rabbit for subcutaneously injected glucose is somewhat below the value generally accepted for the dog.

When glucose is repeatedly administered, at intervals of 1 week, the assimilation limit of rabbits is not influenced appreciably.

TABLE III.

The Influence of Sodium Carbonate (0.5 per Cent Solution) upon the Hyperglycemia and Glycosuria Resulting from the Subcutaneous Injection of Glucose (30 per Cent Solution).

Date.	Rabbit.	Weight.	No. of injection.	Volume of glucose solution injected.	Volume of sodium carbonate injected.	Blood sugar content (in percentages).							Urine.	
						Normal.	Hrs. after injection of glucose.						Volume.	Sugar content.
							½	1	2	3	4	5		
		gm.		*cc.*	*cc.*								*cc.*	*gm.*
July 20	54	2,700	2	44*	130	0.106	0.22	0.215	0.206	0.189	0.194	0.147	700	0.272
" 20	55	3,080	2	50.5*	130	0.104	0.188	0.161	0.141	0.159	0.173	0.169	1000	0.462
" 21	57	2,320	2	41*	120	0.115	0.281	0.272	0.259	0.261	0.23	0.204	1000	0.7
" 28	59	2,460	3	41*	125	0.118	0.335	0.328	0.286	0.26	0.174	0.146	1750	0.567
Oct. 4	65	1,800	1	27†	100	0.115	0.253	0.254	0.21	0.195	0.156	0.138	960	0.068
" 4	66	1,680	1	25.2†	100	0.135	0.274	0.266	0.209	0.185	0.149	0.139	840	0.000
" 6	61	2,840	2	44.4†	130	0.122	0.428	0.423	0.281	0.24	0.142	0.132	940	0.985
" 9	63	1,760	2	28.2†	100	0.129	0.324	0.318	0.242	0.164	0.123	0.12	800	0.083
Average						**0.118**	**0.288**	**0.28**	**0.229**	**0.207**	**0.168**	**0.149**		**0.392**

* Dextrose, Merck.
† Dextrose, Kahlbaum.

TABLE IV.

The Influence of Sodium Chloride (0.364 per Cent Solution) upon the Hyperglycemia and Glycosuria Resulting from the Subcutaneous Injection of Glucose (30 per Cent Solution).

Date.	Rabbit.	Weight.	No. of injection.	Volume of glucose solution injected.	Volume of sodium chloride injected.	Blood sugar content (in percentages).							Urine.	
						Normal.	Hrs. after injection of glucose.						Volume.	Sugar content.
							½	1	2	3	4	5		
		gm.		*cc.*	*cc.*								*cc.*	*gm.*
July 27	55	2,960	3	50.5*	130	0.112	0.353	0.34	0.209	0.181	0.18	0.147	170	0.433
" 27	57	2,280	3	41*	120	0.111	0.343	0.402	0.398	0.296	0.219	0.18	140	0.475
Aug. 1	59	2,360	4	41†	125	0.119	0.311	0.325	0.293	0.224	0.163	0.145	170	0.935
Oct. 13	61	2,580	3	44.4‡	130	0.125	0.306	0.288	0.179	0.147	0.153	0.137	235	0.245
" 18	65	1,860	3	27‡	100	0.118	0.204	0.159	0.14	0.122	0.127	0.125	220	0.000
" 18	66	1,740	3	25.2‡	100	0.139	0.212	0.231	0.183	0.167	0.169	0.16	205	0.000
Average						**0.121**	**0.288**	**0.291**	**0.234**	**0.189**	**0.169**	**0.149**		**0.348**

* Dextrose, Merck.
† Commercial glucose.
‡ Dextrose, Kahlbaum.

The influence of the intravenous injection of sodium carbonate upon the hyperglycemia and glycosuria in normal rabbits, following the subcutaneous administration of glucose is not significant. Wide variations in the response of different animals are exhibited. The results are in agreement with those of a previous paper,[3] in which the data indicated that the sugar-regulating mechanism of a normal animal is not usually influenced by the intravenous injection of small quantities of sodium carbonate.

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[3] McDanell and Underhill, *J. Biol. Chem.*, 1917, xxix, 227.

STUDIES IN CARBOHYDRATE METABOLISM.

XX. NEW EXPERIMENTS UPON THE MECHANISM OF SALT GLYCOSURIA.

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It was noted by Bock and Hoffman in 1871 that large amounts of a 1 per cent solution of sodium chloride, injected into the arterial circulation of rabbits, gives rise to diuresis, followed by glycosuria. Külz demonstrated that similar effects follow the introduction of other sodium salts—the acetate, carbonate, valerianate, and succinate—as well as the chloride. Martin Fischer confirmed these results. Moreover, he showed that, when glycosuria has been established, it may be arrested by subsequent injections of a mixture of the sodium salt and calcium chloride. A reappearance of sugar in the urine is brought about by the replacement of the calcium solution by that of a sodium salt. Fischer also found that the rapidity with which sugar appears in the urine varies directly with the concentration of the salt solution employed, that the degree of diuresis bears no definite relation to the quantity of sugar excreted, and that albuminuria is a frequent accompaniment of this glycosuria. Results similar to those of Fischer were obtained by Brown. Owing to the fact that the latter investigator used an anesthetic which of itself is capable of producing glycosuria, Fischer discounts the value of Brown's results. Later, Fischer (1905) observed that when sodium salts are injected directly into the arterial system (into the axillary artery, hence through the vertebral arteries to the spinal bulb) glycosuria occurs sooner than after injections into the venous circulation. From these facts he concluded that, in the glycosuria provoked by the introduction of sodium salts into the circulation, two factors are involved: one, an action upon the kidney by which diuresis is induced; the second, an influence upon the diabetic center in the spinal bulb, which causes glycosuria.

Underhill and Closson demonstrated a difference between the glycosuria following the introduction of solutions of sodium chloride into the venous and into the arterial circulation of the rabbit. They attribute the glycosuria in the former instance, which is accompanied by polyuria, to an increased permeability of the kidney. This permeability may be decreased by the injection of a mixture of sodium chloride and calcium chloride, as

indicated by the temporarily decreased flow of urine and diminished or inhibited excretion of sugar. Also, they found this form of glycosuria to be accompanied by a hypoglycemia, which gave place to normal blood sugar, or even hyperglycemia, when the excretion of sugar in the urine is inhibited by the injection of a calcium salt. On the other hand, injection of sodium chloride into the cerebral arterial circulation induces glycosuria with no polyuria, but with an accompanying hyperglycemia. They believe that, in the latter instance, the increased content of sugar in the blood may be referred to disturbances of respiratory processes, dyspnea, provoked by the introduction of sodium chloride.

Further work upon this subject led McGuigan and Brooks to state: "The permeability of the kidney is of small import in the mechanism of experimental glycosuria."

Underhill and Kleiner, considering this conclusion not justified by the data given, repeated certain of the experiments of McGuigan and Brooks, and as the result, say: "Renewed investigation has afforded no occasion to modify the conclusions already reached regarding the mechanism of salt glycosuria. Under appropriate conditions in the rabbit glycosuria due to renal permeability induced by sodium chloride injections can be inhibited by injections of calcium chloride. If calcium chloride is appropriately introduced glycosuria fails to be evinced even when free sugar is injected in addition."

More recently Hirsch failed to obtain glycosuria, without an accompanying hyperglycemia, after intravenous injections of sodium chloride. Since his results are diametrically opposed to those obtained by Underhill and his coworkers, the subject of the mechanism of salt glycosuria has been submitted to renewed investigation. Particular interest attaches to the question whether at the period of glycosuria provoked by salt infusion there is augmentation of the level of blood sugar. It is evident that, if blood sugar content is not increased under these circumstances, as asserted by Underhill and his coworkers, a change in renal permeability must be assumed. Otherwise, in the absence of hyperglycemia, there is no obvious explanation for the existing glycosuria.

Methods.

These experiments were performed upon full-grown, well fed rabbits, without anesthesia. The "bootjack" rabbit holder used in this laboratory has been described elsewhere.[1] The blood sugar was determined by the Lewis-Benedict method. Haldane's procedure for the estimation of hemoglobin[2] was followed. Benedict's qualitative reagent was used in testing for

[1] McDanell, L., *J. Lab. and Clin. Med.*, 1916, i, 804.
[2] Haldane, J., *J. Physiol.*, 1901, xxvi, 497.

sugar in the urine. The solution of sodium chloride was injected continuously, under pressure, into the marginal vein of an ear. Samples of blood for the determination of blood sugar and hemoglobin were obtained from a vein of the other ear. The bladder was emptied at intervals by pressure through the abdominal wall.

The results of seven experiments are given in the accompanying table. From these data it may be seen that *the intravenous injection of* M/2 *sodium chloride invariably induced glycosuria within a period of 15 to 20 minutes.* The volume of solution required to provoke the appearance of sugar in the urine varied from 130 cc. in Experiment 4 to 224 cc. in Experiment 3, the average of the six experiments being 176 cc.

The glycosuria observed was not due to hyperglycemia, for, although the hypoglycemia noted by Underhill and Closson was not in evidence, *the highest blood sugar content exhibited at the end of an injection was 0.14 per cent* (Experiment 6). Under these circumstances, *sugar in the urine cannot be due to a condition of hyperglycemia.* It is therefore apparent that the glycosuria induced must be ascribed to a renal factor, as originally proposed by Underhill and Closson.

In two instances (Experiments 2 and 3) a hyperglycemia was found 1 hour after the end of the injection. Rabbit 79 (Experiment 2) was prostrated as the result of the injection, but recovered rapidly. In Rabbit 80 (Experiment 3) which developed a hyperglycemia of 0.286 per cent 1 hour after the injection, there was much greater prostration, in fact a paralysis, from which the animal did not recover. The prostration appears to have been due to the poisonous effects of a large excess of the sodium salt. Wherever such a condition was encountered in our experiments, hyperglycemia accompanied it. In this connection, it is to be noted that Rabbit 80 excreted a relatively small amount of urine, and also that the fall in the percentage of hemoglobin was greater than with any of the other rabbits.

The figures for blood sugar cannot be accounted for by mere dilution of the blood as a result of the salt solution administered. This may be seen from the values for the hemoglobin, determined before and just after the injection.

TABLE.

Time.	Solution injected.	Urine excreted.	Hemoglobin.	Blood sugar.	Remarks.
	In last interval of time.				
	Experiment 1. Rabbit 78, 2,700 gm. October 24, injection of M/6 sodium chloride.				
	cc.		*per cent*	*per cent*	
10.00			55	0.115	
10.10					Injection begun.
10.25	100				No sugar.
10.40	94				" "
10.55	114				" "
11.10	71				" "
11.25	102				" "
11.40	77				" "
11.55	112				" "
12.10	83				" "
12.25	100				" "
12.40	82				" "
12.55	128				" "
1.10	85				" "
1.25	129				Sugar.
1.35	44				"
	1,321				Injection stopped.
1.45			50	0.138	
3.00			56	0.116	Rabbit had poor use of muscles when first taken from board. Recovered rapidly.
	Experiment 2. Rabbit 79, 2,240 gm. October 26, injection of M/2 sodium chloride.				
9.40			55	0.119	
9.47					Injection begun.
9.53	60				
9.57	102	55			No sugar.
10.02	34	45			Sugar.
10.07	21	30			"
10.12	23	31			"
10.17	22	31			"
10.22	31	32			"
	293	224			Injection stopped.
10.25			50	0.136	
11.25			60	0.228	Rabbit partially paralyzed. Recovered.

TABLE—*Continued.*

Time.	Solution injected. (In last interval of time.)	Urine excreted. (In last interval of time.)	Hemoglobin.	Blood sugar.	Remarks
					Experiment 3. Rabbit 80, 2,360 gm. October 27, injection of M/2 sodium chloride.
	cc.		*per cent*	*per cent*	
9.45			42	0.119	
9.53					Injection begun.
9.58	60				
10.03	54	20			No sugar.
10.08	54	16			Trace of sugar.
10.13	56	14			Sugar.
10.18	44	8			"
10.23	31	6			"
	299	64			Injection stopped.
10.25			32	0.116	
11.25			43	0.286	Rabbit paralyzed. Dead next morning.
					Experiment 4. Rabbit 81, 2,580 gm. October 28, injection of M/2 sodium chloride.
10.00			58	0.111	
10.08					Injection begun.
10.13	58	16			No sugar.
10.18	34	19			" "
10.23	38	45			Sugar.
10.28	37	70			"
	167	150			Injection stopped.
10.30			56	0.121	
11.30			58	0.136	Rabbit in excellent condition at end of experiment.

TABLE—*Concluded.*

Time.	Solution injected. (In last interval of time.)	Urine excreted. (In last interval of time.)	Hemoglobin.	Blood sugar.	Remarks.
					Experiment 5. Rabbit 82, 2,740 gm. October 31, injection of M/2 sodium chloride.
	cc.		*per cent*	*per cent*	
10.00			53	0.104	
10.14					Injection begun.
10.19	62	3			No sugar.
10.24	42	16			" "
10.29	39	37			" "
10.34	29	45			Sugar.
10.37	40	45			"
	212	146			Injection stopped.
10.42			50	0.105	
11.42			56	0.107	Rabbit in good condition at end of experiment.
					Experiment 6. Rabbit 83, 2,500 gm. November 1, injection of M/2 sodium chloride.
2.00			56	0.125	
2.11					Injection begun.
2.16	80	22			No sugar.
2.21	52	50			Trace of sugar.
2.26	24	55			Sugar.
2.29	14	23			"
	170	150			Injection stopped.
2.30			55	0.14	
3.30			62	0.122	Rabbit in good condition at end of experiment.
					Experiment 7. Rabbit 84, 2,380 gm. November 2, injection of M/2 sodium chloride.
11.00			47	0.108	
11.10					Injection begun.
11.15	65	5			No sugar.
11.20	43	16			" "
11.25	36	26			" "
11.30	37	28			Sugar.
11.32	14	24			"
	195	99			Injection stopped.
11.37			43	0.123	
12.40			48	0.125	Rabbit in good condition at end of experiment.

The fact that Hirsch failed to obtain glycosuria, without hyperglycemia, from injections of solutions of sodium chloride is not difficult to explain. In the first place, his injections were not continuous. In this and other laboratories, positive results have been obtained only with continuous injections. Further, Hirsch introduced in a given period of time an insufficient amount of 0.85 per cent and 2.5 per cent solutions. This fact is of added significance because many of his rabbits were much larger than those used here.

Hirsch attributes the sugar in the urine, caused by *dilute* salt solutions, to "emotional glycosuria" in consequence of tying the rabbits in a holder. We are unaware of the methods practiced by Hirsch for the accomplishment of this purpose, but the procedure employed in this laboratory is never productive of hyperglycemia or glycosuria. As an illustration of this point may be cited the experience with Rabbit 78 (Experiment 1). This animal remained on the rabbit board for more than 3 hours, but exhibited no significant change in blood sugar content in spite of long continued infusion of salt solution. Conditions were ideal for the production of "emotional" hyperglycemia and glycosuria, which, however, failed to occur.

We have had no experience with injections of sodium chloride solutions stronger than M/2. It seems evident, however, that the hyperglycemia and glycosuria, observed by Hirsch with 10 per cent solution, are to be attributed to the toxic influence which must necessarily be exerted by such a strong salt solution. The data obtained under these circumstances bear no relation to the question under investigation.

CONCLUSIONS.

Further experiments upon the mechanism of salt glycosuria afford no support for the contention of Hirsch that renal permeability is not an important factor in the condition under discussion. On the contrary, the data here given are in agreement with those of Underhill and Closson, and Underhill and Kleiner, previously reported. The results indicate that the glycosuria caused by the introduction of dilute solutions of sodium chloride into the venous circulation is due to an increased permeability of the kidney.

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STUDIES OF AUTOLYSIS.

V. THE INFLUENCE OF BILE ON AUTOLYSIS.

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In a recent article[1] Tatum describes the marked disintegration of tissues immersed in bile or its salts. In the peripheral zone, where there was evident penetration by the bile, cytolysis was apparent within 4 hours or less, the nuclear material and cytoplasm showed alterations in staining reactions, and in some cases disintegration was so complete as to leave only connective tissue structures. The facts developed in this study are of importance physiologically and pathologically. They suggest in a striking way the possibilities of tissue damage which may result from contact with bile. At the same time the mechanism by which this cytolysis is induced does not appear to us to have been conclusively established by the author. The conclusion reached was that this disintegration of tissue cells by bile was correlated with autolysis. "*This cytolysis is produced by virtue of the coenzyme or activating action of the constituents of bile on the autolytic enzymes or processes.*" It should be pointed out that this conclusion is chemical, while the observations leading to it were wholly histological. Furthermore, if the conclusion is valid it establishes an important chemical mechanism connected with the autolytic process about which there still exists some doubt and for which no direct evidence has yet been found; *i.e.*, activation of the enzymes.

In the data presented by Tatum the following points appear to us of significance. First, the reaction described is one of tremendous speed compared with normal autolysis. A block of tissue

[1] Tatum, A. L., *J. Biol. Chem.*, 1916, xxvii, 243.

disintegrates twenty times as fast in bile as it does in dilute acid. Yet dilute acid is by far the best autolytic accelerator hitherto found. If the action of bile is one of enzyme activation it must produce an amount of active enzyme many times that found in normal tissue or in tissue of optimal acidity. Such an enormous alteration of activity leads us to question whether the explanation given is sound. In the second place, the fact that fibrin and heat-coagulated tissues were not dissolved in bile while the fresh tissues were does not necessarily imply that enzymes are required in the process. The solvent action of the bile salts on the proteins themselves has not been ruled out. Third, the order of rapidity of disintegration follows to a striking degree the order of rapidity of autolysis. The glands like liver and spleen go to pieces rapidly, while muscle tissue disintegrates slowly. There may indeed be some fundamental connection between the disintegration of these tissues and the ease with which their proteins hydrolyze without involving any further connection between the action of bile and the autolytic enzymes. Finally, there is a certain lack of distinction between the terms cytolysis and autolysis as used by the author, which has, we believe, led to the errors of interpretation. Autolysis was the term given by Jacoby[2] to the process of tissue disintegration involving deep-seated hydrolysis of the proteins. It was so used by Hedin, Rowland, Preti, and a score of others following Jacoby's work. It has carried the same implications up to the present time, with occasional broadening of the term to include other hydrolyses going on in postmortem tissue, such as the digestion of fats or glycogen. Where not otherwise specified the term autolysis signifies tissue autoproteolysis. On the other hand, cytolysis is a morphological term; it may or may not involve deep-seated chemical change back of the cell disintegration. It is used to describe the laking of the red corpuscles by water or other hemolytic agents, it is also used to describe the cell disintegration of a tissue after death. The one is probably a physical phenomenon, the other is certainly chemical. In certain cases cytolysis and autolysis are synonymous, in others they are not.

The results of Tatum's investigations do not constitute a proof of the activation of an enzyme, since no test of enzyme activity

[2] Jacoby, M., *Z. physiol. Chem.*, 1900, xxx, 174.

was made. The data are subject to interpretation in a variety of other ways, and indeed it may be questioned whether autolysis is involved at all in the process described. The microscopical picture of cytolysis is inadequate to demonstrate either autolysis or enzyme activation. For this reason we have subjected the problem to a definite chemical test, using the technique described in previous papers,[3] and estimating the speed and extent of autolysis by the amino-acids produced. At the outset we assumed that bile did accelerate autolysis, but were skeptical of enzyme activation. Our first experiment was designed therefore specifically to determine whether activation took place by following the rate of digestion of some foreign digestible protein like gelatin (see Table VI). To our surprise we found that bile does not significantly change the rate of autolysis of such tissues as liver, heart, spleen, kidney, and thymus. It cannot function therefore either as an activator or as a coenzyme in the autolytic mechanism.

Experiment I. The Effect of Bile on Autolysis of Various Tissues.—The tissues were ground, sieved, and made up to 250 cc. as in previous experiments. Bile was present where indicated in the tables. Digestion was estimated by formol titration of the amino-acids in the trichloroacetic acid filtrates. Bile caused marked alterations in the appearance of the protein precipitates, but did not interfere seriously with the end-points of titrations. The figures in the tables representing digestion are given in cc. of 0.2 N NaOH per 25 cc. of filtrates.

TABLE I.

Pig Liver; Mixed Pig and Beef Bile.

	Days.				Gain.
	0	2	5	16	
I. Control	0.40	2.35	2.90	3.45	3.05
II. " + 100 cc. bile	0.45	1.15	1.55	2.10	1.55
III. " + 25 " "	0.45	1.80	2.30	3.05	2.60
IV. " + 6 " "	0.50	2.10	2.80	3.45	2.95

In this experiment we find definite inhibition of autolysis in the higher concentrations of the bile.

[3] Bradley, H. C., and Taylor, J., *J. Biol. Chem.*, 1916, xxv, 261.

TABLE II.

Pig Heart; Pig Kidney; Beef Bile.

	Days.				Gain.
	0	2	5	16	
I. Control, heart muscle	0.30		0.80	1.05	0.75
II. " + 100 cc. bile	0.35	0.60	0.65	0.90	0.55
III. " + 25 " "	0.35	0.65	0.85	1.10	0.75
IV. " + 6 " "	0.30		0.85	1.20	0.85
V. " in 0.02 N HCl	0.30	0.95	1.40	1.95	1.65
I. Control, kidney	0.50	0.95	1.00	1.25	0.75
II. " + 100 cc. bile	0.60	1.00	1.35	1.80	1.20
III. " + 25 " "	0.50	1.00	1.30	1.80	1.30
IV. " + 6 " "	0.50	1.00	1.15	1.30	0.80
V. " in 0.02 N HCl	0.50		3.05	3.70	3.20

Bile has no significant effect on cardiac muscle. The stronger solutions appear to inhibit slightly, the weaker to accelerate. Acidity produces definite increase of autolysis. In the case of the kidney, higher concentrations of bile appear to accelerate the autolysis and lead to a higher final level of equilibrium. The effect is of little significance, however, when compared with the 300 per cent increase of autolysis in the presence of acid. This small increase of autolysis in the presence of bile we have found to be the rule with kidney and thymus, and it is sometimes found in the spleen. It clearly has no relation to the twentyfold rate of cytolysis reported by Tatum over the cytolysis in the presence of acid.

The cause of this regular increase of autolysis in some tissues we thought might be accounted for by the bile proteins introduced. Precipitating the proteins from 100 cc. of bile and adding the washed precipitate to a tissue digest failed to produce a measurable effect. It is possible that the alcohol-precipitated protein was sufficiently altered to fail to digest where the protein in solution was digestible. It is more probable that there are sufficient traces of fats of the lower fatty acids in some of the tissues to produce appreciable amounts of fatty acids under the accelerating influence of bile on lipolysis. These water-soluble acids would accelerate the rate and increase the extent of proteolysis.

TABLE III.

Beef Kidney; Beef Bile.

	Days.			Gain.
	0	2	7	
I. Control	0.40	0.75	0.90	0.50
II. " + 100 cc. bile	0.60	1.10	1.40	0.80
III. " + 25 " "	0.45	0.90	1.15	0.70
IV. " + bile protein	0.45	0.75	0.90	0.50
V. " in 0.02 N HCl	0.45	2.75	3.45	3.00

TABLE IV.

Beef Spleen; Calf Thymus; Beef Bile.

	Days.				Gain.
	0	2	7	16	
I. Control, beef spleen	0.45	2.15	2.55	2.80	2.35
II. " + 100 cc. bile	0.60	1.80	2.50	3.10	2.50
III. " + 25 " "	0.55	2.10	2.75	3.15	2.60
IV. " + 6 " "	0.55	2.30	2.70	3.10	2.45
V. " in 0.02 N HCl	0.60	3.10	3.95	4.10	3.50
I. Control, calf thymus	0.75	1.40	1.90	2.10	1.35
II. " + 100 cc. bile	0.70	1.55	2.25	2.70	2.00
III. " + 25 " "	0.70	1.60	2.25	2.70	2.00
IV. " + 6 " "	0.70	1.45	2.10	2.50	1.80
V. " in 0.02 N HCl	0.75	2.60	3.15	3.40	2.65

TABLE V.

Beef Spleen; Calf Thymus; Beef Bile.

	Days.			Gain.
	0	3	7	
I. Control, beef spleen	0.50	1.75	2.20	1.70
II. " + 100 cc. bile	0.75	2.00	2.35	1.60
III. " + 25 " "	0.65	1.95	2.30	1.65
IV. " in 0.02 N HCl	0.55	3.50	3.65	3.10
I. Control, calf thymus	0.40	1.10	1.35	0.95
II. " + 100 cc. bile	0.60	1.30	1.85	1.25
III. " + 25 " "	0.45	1.20	1.65	1.20
IV. " in 0.02 N HCl	0.50	2.65	3.40	2.90

Comparing Tables IV and V we see that in one case the spleen is slightly accelerated and increased in its autolysis by bile, in the other the reverse is true. If the phenomenon were one involving activation we should look for uniformity. Its variability suggests some factor like fat or ester content which is likely to vary under different nutritional conditions.

Experiment II. Bile and the Digestion of Foreign Proteins.—Gelatin was chosen as a typical foreign protein normally digested by the liver proteases. Activation of the enzymes should lead to a more rapid hydrolysis of gelatin.

TABLE VI.

Pig Liver; Gelatin; Dried Bile.

	Time.						Gain.
	0	4 hrs.	11 hrs.	1 day.	6 days.	14 days.	
I. Control	0.55	0.90	1.20	1.50	2.70	2.90	2.35
II. " + gelatin	0.60	1.10	1.50	2.00	3.55	3.90	3.30
III. " + 20 gm. bile	0.90	1.15	1.30	1.45	2.20	2.50	1.60
IV. Control + bile + gelatin	0.95	1.20	1.40	1.65	2.50	2.90	1.95
V. Control + 5 gm. bile	0.70	0.90	1.10	1.40	2.30	2.50	1.80
VI. Control + bile + gelatin	0.80	1.05	1.35	1.70	2.80	3.10	2.30
VII. Control in 0.02 N HCl	0.55	1.15	1.70	2.10	3.45	3.70	3.15
VIII. Control + gelatin in HCl	0.65	1.15	1.80	2.60	4.20	4.55	3.90

Analysis of the figures shows the following.

Gelatin in the control digests 0.95 cc.
" with 20 gm. of bile present digests 0.35 cc.
" " 5 " " " " " 0.50 "
" " HCl present digests 0.75 cc.

It is clear that bile retards the digestion of gelatin, as it does the digestion of the liver proteins. There is no suggestion of an activation phenomenon.

DISCUSSION.

The experiments emphasize the caution which must be exercised in interpreting histological changes in terms of chemical reactions without at the same time demonstrating the presence of such reactions. It is evident, too, from the combined data presented by Tatum and by us that a sharp distinction must be made between autolysis and cytolysis. The disintegration of cell structures may take place without accompanying autolytic changes, as when the red corpuscles are laked with water, bile salts, saponin, or toluene. It may take place with no disturbance of the autolytic mechanism, except perhaps to hasten the death of the tissue and so the onset of autolysis. Cytolysis of this kind is described in Tatum's paper—blocks of fresh liver, spleen, kidney, etc., immersed in bile. Finally cytolysis may be the direct result of autolysis, as when postmortem changes occur in the same tissues under normal conditions, or where blocks of these tissues are immersed in acid of the proper concentration to accelerate autolysis.

The mechanism of bile cytolysis is not clear in the cases presented by Tatum beyond the fact that it is not correlated with an increased rate of autolysis. It may be due to the solvent effect of the bile salts upon the cell lipoids. It may be due to the greater dispersion of structure colloids in the lowered surface tension of bile solutions, and thus have an apparent solvent effect. It is well known that bile dissolves the visual purple of the retina as a more highly dispersed colloid, and the two phenomena are not unlike.

SUMMARY.[4]

1. Autolysis does not parallel the rapid cytolysis of tissues immersed in bile or its salts. Bile does not accelerate the autolysis of liver, spleen, kidney, thymus, and heart muscle to a significant degree.

[4] We have submitted our manuscript to Dr. Tatum for his comment, and find that he has independently been engaged in much the same line of experimentation. His results, too, are in substantial agreement with those presented here.

2. Bile does not activate the enzymes associated with the phenomena of autolysis in the tissues investigated, nor does it act as a coferment.

3. The cytolytic effect of bile on these tissues must therefore be a process distinct from autolysis.

THE USE OF COTTON SEED AS FOOD.*

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The following quotations from recent literature relating to the use of cotton seed products as food serve to illustrate the general problem of so called cotton seed meal injury or toxicity:

"Cotton seed meal is one of the most valuable feedstuffs at the command of the American stockman. After the animal has digested it, the value of the residue as fertilizer is about three-fourths the original value of the meal. The United States uses only part of the cotton seed meal which it produces, and one of the reasons which prevent a larger domestic consumption of this by-product of the cotton industry is the danger that sickness and death may follow its use. Cattle fed for more than 90 to 120 days on a heavy cotton seed meal ration (6 pounds or more per head daily) become lame, and their eyes discharge freely, blindness often resulting. Deaths may occur, especially in young animals. Pigs are peculiarly susceptible to the effects of cotton seed meal, possibly because they are usually fed a larger quantity of the meal in proportion to their body weight. In feeding pigs, symptoms of sickness may appear at any time after 3 weeks of feeding, and deaths frequently occur with little warning. Various systems of feeding cotton seed meal to pigs have been devised. Some of them appear to minimize its danger somewhat, but none of them prevent it entirely. This product, therefore, can not be regarded as a safe feed for pigs in the combinations in which it has heretofore usually been fed."[1]

Referring to the experiments in feeding cotton seed meal which

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Rommel, G. M., and Vedder, E. B., *J. Agric. Research*, 1915, v, 489.

have been made by various agricultural station workers, Wells and Ewing[2] summarize:

"The results of such experiments do not agree entirely and few absolute conclusions can be drawn from them. They indicate, however, that swine—particularly young pigs—calves, sheep, horses, cows, steers, dogs, cats, guinea pigs, rabbits, fish, poultry, and other animals may be injured by eating cotton seed meal. Some of the smaller animals, such as pigs and calves, seem to be more susceptible to its injury than cows, steers, and similar animals. This, however, may have been due to their youth, or, more probably, to a consumption of larger quantities of meal in proportion to their live weight. When the meal was fed in connection with pasturage, or when it had been steamed, boiled, or fermented, or when fed with mineral matter, particularly iron compounds, it often seemed to exert no apparent injury to pigs even when fed in rather large quantities. The injury resulting from the feeding of cotton seed meal to stock has been attributed to: (*a*) the oil in the meal; (*b*) its crude fiber; (*c*) excess of nitrogen and perverted metabolism; (*d*) the action of bacteria and molds; (*e*) presence of betain, cholin, or other alkaloids, and to gossypol; (*f*) to injurious phosphorous compounds; (*g*) to a protein group containing loosely bound sulphur, which interferes with normal iron metabolism; to worms and certain other causes, which, perhaps, are not of sufficient importance to merit discussion here."

Until quite recently there has been little, if any, appreciation of the possibility that so called cotton seed injury may be associated with a lack of some essential dietary component rather than attributable solely or even in part to the presence of a positively harmful ingredient of the seed. According to present day criteria a "balanced" or "physiological" ration must represent something more than an adequate quota of calories including no less than a certain minimal proportion of protein, along with inorganic salts and, perhaps, "roughage." The protein must be suitable in quality, so as to furnish a sufficient yield of all the needed amino-acids; the inorganic nutrients must be both quantitatively and qualitatively appropriate; and evidently certain vitamines (including those soluble in some of the natural fats, *e.g.*, butter fat or cod liver oil, and also those soluble in water which appear to be widely distributed in the active cells of plants and animals) are needed, even if the absolute quantity requisite

[2] Wells, C. A., and Ewing, P. V., *Georgia Agric. Exp. Station, Bull. 119*, 1916.

is small. Furthermore, positively toxic substances must be absent or at least detoxicated in some way.

Basing their decision upon the similarity of the symptoms of the so called cotton seed poisoning of pigs with those seen in the "deficiency disease," beri-beri, Rommel and Vedder have come to the conclusion that cotton seed injury to pigs is probably due to some *deficiency* in the ration. Precisely wherein the shortcoming lies is not indicated by them, though they offer the following explanation:

> "The grain with which the cotton seed meal is most frequently combined is corn. Corn is notoriously deficient as a single feed for animals, and it must be properly balanced to be fed satisfactorily. The excellent results in feeding pigs which can be obtained from rations of corn meal and skim milk or other animal products, such as tankage, blood meal, fish meal, etc., are out of all proportion to the facts indicated by the conventional chemical analyses of protein, carbohydrates, and fat. When corn meal is fed with cotton seed meal, a combination is made of two feeds both of which are deficient."

To avoid confusion of the questions at issue it must be noted that cotton seed products for feeding purposes are available in several commercial forms. Cotton seed *kernels* are obtained when the whole cotton seed is decorticated and freed from most of the hulls. Cotton seed *meal* is the term applied to the ground cotton seed cake from which most of the oil has been pressed. Cotton seed *flour* is prepared by finely grinding and sifting the meal, whereby the lint, hulls, etc., are removed more completely than from cotton seed meal.

According to information received from the Bureau of Chemistry at Washington two processes are commonly employed in preparing cotton seed *meal*. In the first process the seeds are decorticated, ground, and then subjected in kettles to the action of live steam for about $\frac{3}{4}$ of an hour. The hot mass is freed from most of the oil by means of hydraulic presses and the resulting press cake is then ground to a meal. In the second process the oil is expressed from the seeds by means of Anderson expellers whereby the meal becomes heated. The residue is then ground as in the first process. The second procedure is frequently called the cold process. We are informed that nearly all of the mills in this country use the method employing live steam; and we do

not know of any mill at present operating which does not use heat in some form. Heat is regarded as necessary in order to obtain satisfactory yields of oil.

Marchlewski[3] isolated from the "foots" from cotton seed oil a substance which he named gossypol. Withers and Carruth[4] have isolated this from cotton seed *kernels*, by extraction with fat solvents, and they report it to be highly toxic to rabbits, guinea pigs, rats, and pigs. They state that the ether-extracted *kernels* are rendered non-toxic by removal of gossypol. According to Withers and Carruth, "cotton seed meal and flour were found toxic to rabbits but the flour produced no ill effects on rats." The unlike toxicity to rats is explained by the variation in alteration or removal of gossypol in the manufacture of the *meal* from the *kernels*. Withers and Carruth believe that the deficiency theory will not explain the quick deaths of pigs and other animals fed on cotton seed *meal*, although with all other dietary factors adequate, the toxicity may be more or less overcome.

Employing the technique which we have followed for some years in our nutrition experiments with rats, Richardson and Green[5] have undertaken an investigation on the efficiency of cotton seed meal and cotton seed flour as a food for promoting growth, development, and reproduction of the albino rat. Their results led them to conclude that failures to grow or be maintained on diets containing either cotton seed meal or cotton seed flour as the chief or sole source of protein were in each instance attributable to a lack of some (non-protein) essential, such as certain inorganic salts or the growth-promoting substances present in butter fat and the water-soluble vitamine of milk, rather than to any apparent toxicity. When butter fat and "protein-free milk" or whole milk powder, which also contains the vitamines in question, were added to cotton seed flour, satisfactory nutrition was maintained over long periods. Albino rats were still alive after nearly a year on diets containing as much as 33 to 50 per cent cotton seed flour. Richardson and Green state that "animals fed on cotton seed meal extracted with ether, according to the

[3] Marchlewski, L., *J. prakt. Chem.*, 1899, lx, 84.

[4] Withers, W. A., and Carruth, F. E., *J. Agric. Research*, 1915, v, 261.

[5] Richardson, A. E., and Green, H. S., *J. Biol. Chem.*, 1916, xxv, 307.

method of Withers, have shown no nutritional advantages over those rats fed on the unextracted meal." In fact, failure sometimes occurred sooner on the extracted meal diets owing, it is suggested, to the removal of the fat-soluble growth-promoting vitamine such as is present in milk fat. This should be kept in mind in connection with the contradictory statement of Withers and Carruth that "cotton seed kernels are rendered less toxic by the partial extraction of gossypol and non-toxic by a more nearly complete extraction of it." Richardson and Green conclude that "cotton seed meal does not contain sufficient minerals for growth, is not actively toxic, contains efficient protein, and perhaps fat-soluble growth-promoting substances, similar to those of butter fat, but in less adequate quantities."

We are not aware that any case of injury to man has been reported from the use of cotton seed flour as food.

EXPERIMENTAL.

To ascertain whether the cotton seed proteins are, like some proteins from maize[6] notably deficient for the purposes of nutrition we have conducted feeding experiments on white rats for which cotton seed proteins furnished practically all of the food nitrogen and for which the other essential dietary components were supplied by adding to the products to be tested a suitable mixture of "protein-free milk," butter fat, and starch which, with the addition of adequate protein, has been shown in hundreds of our experiments to be sufficient for perfect growth. In this way we have found that satisfactory growth can be made by rats when either cotton seed *globulin*, or the total cotton seed protein precipitated from alkali extracts of cotton seed meal, is employed without significant amounts of other protein in the mixture.[7] The globulin was prepared by the salt extraction method[8] and represents purified protein soluble in dilute salt solution but

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xiv, p. xxxi; Osborne, T. B., *Science*, 1913, xxxvii, 185; Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325; 1914, xviii, 1; 1916, xxv, 1.

[7] The results of these and other experiments included in this paper were reported to the Society for Experimental Biology and Medicine, May 24, 1916; see *Proceedings*, 1916, xiii, 147.

[8] Osborne, T. B., and Voorhees, C. G., *J. Am. Chem. Soc.*, 1894, xvi, 778.

insoluble in water. The *total alkali-soluble protein* was prepared as follows.

Cotton seed *flour* was treated with ten times its weight of 0.4 per cent potassium hydroxide solution, filter paper pulp, which had been thoroughly extracted with the same dilute alkali, added until a thick mass resulted, and the solution pressed out with a hydraulic press. The extract was then filtered through a dense felt of paper pulp, previously washed with dilute alkali. To the clear filtrate 1 per cent hydrochloric acid was added until the protein separated sharply. The precipitate was then filtered out and washed with 50 per cent alcohol, until the washings were practically free from chlorine, digested twice with liberal quantities of 92 per cent alcohol, dehydrated with absolute alcohol, washed finally with ether, and then dried over sulfuric acid. The ash- and moisture-free preparation (dried at 110°) contained 16.98 per cent of nitrogen.

Results of feeding experiments with these products are given in the Appendix, Chart I.

In order to test the nutritive value of the total proteins as they occur in the seed, the possible lack of vitamines and inorganic salts was provided for by supplying butter fat and "protein-free milk," which we have already demonstrated to be adequate sources of all needed inorganic food ingredients and vitamines. Cotton seed *flour* was obtained from the Schulenburg Oil Mill, Schulenburg, Texas. Our samples of cotton seed *meal* were obtained by mixing samples collected for analysis by this Station and therefore were assumed to be representative of the ordinary commercial product; or they were taken from a bag of the meal purchased in the open market.

The growth curves in Chart II leave no doubt as to the adequacy of the proteins of the cotton seed *meal* or *flour* for the growth of rats. No signs of toxicity exhibited by noticeable effect on growth have been observed with any of these rations. On this diet Rat 3631 gave birth to six young which have grown vigorously.

In discussing the comparative nutritive value of different proteins in growth and the problem of the protein minimum[9] we have emphasized the fact that the comparative poverty of a protein in one or more essential amino-acid groups may not mani-

[9] Cf. Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351; 1915, xxii, 241; 1916, xxvi, 1.

fest itself if the supply of protein in the ration is so abundant as to be considerably in excess of the actual needs. On a more restricted plane of protein intake the "law of minimum" may manifest itself more conspicuously; accordingly, rats make quite unlike growth on the *same* (restricted) intake of such different proteins as casein and lactalbumin though they show quite similar (maximum) increments of weight when the proteins are furnished in greater abundance.

Our "standard ration" for rats contains about 16 per cent of protein in a mixture yielding about 5 calories per 1 gm. of food. With a few proteins, notably lactalbumin, almost perfect growth can be secured for a considerable period when their content in the ration is no greater than 9 per cent. Other proteins supplied in corresponding proportions exhibit their nutritive inferiority by falling behind in growth-promoting capacity. The excellent "quality" of the cotton seed proteins as a whole is attested by the satisfactory growth made on diets furnishing the equivalent of only 9 per cent of protein ($N \times 5.4$)[10] (see Chart III, Rats 3350 and 3368). Even with 6 per cent of the protein considerable growth ensued (see Rats 3415, 3420).

The high comparative nutritive value of the cotton seed proteins was demonstrated by using cotton seed *flour* as a supplement to such decidedly inferior protein concentrates as "corn gluten,"[11] the protein of which yields only minimal quantities of lysine and tryptophane (see Chart III). Good results were likewise obtained when cotton seed *flour* was used as a supplement to distillers' grains, a commercial product with an amino-acid content not very different from that of the corn gluten; and to "vegetable albumin flour," a product rich in gliadin,—a protein deficient in lysine. Here again no failures attributable to toxicity of the cotton seed *flour* were observed (see Chart III, Rats 3514, 3523, 3515, 3525, 3569).

With regard to the presence of various vitamines in cotton seed products our experience corroborates the findings of Richard-

[10] The limitations of the method of experimentation here employed have already been discussed; see Osborne and Mendel, *J. Biol. Chem.*, 1915, xxii, 246.

[11] Cf. Osborne and Mendel, *J. Biol. Chem.*, 1914, xviii, 1; also 1917, xxix, 69.

son and Green. When diets were prepared with "artificial protein-free milk," *i.e.*, without the water-soluble vitamines other than those contained in the cotton seed, normal gain in body weight was still possible. The food mixtures contained:

	A.	B.
	per cent	*per cent*
Cotton seed flour	41.0	
Cotton seed meal		49.1
"Artificial protein-free milk"	29.5	28.0
Butter fat	18.0	18.0
Starch	3.5	4.9
Lard	8.0	

The "artificial protein-free milk" has already been described.[12] Aside from the lactose present this consists of salts obtained from carefully purified chemicals. The mixture has been found to furnish the mineral ingredients suitable for excellent growth provided some other source of vitamines is present. Without these, prolonged growth very rarely is secured, as we have found in many trials. In the above diet the growth-promoting (fat-soluble) vitamine has been supplied by butter fat. In view of the satisfactory growths obtained with these diets in the absence of any other source of this accessory, as manifested by the records in Chart IV in the Appendix, the cotton seed must furnish the water-soluble vitamine in considerable abundance. This has now been found true of many other seeds by McCollum and his coworkers.

McCollum, Simmonds, and Pitz[13] have lately reported cotton seed *oil* prepared by ether extraction to be toxic; whereas the commercial bleached *oil* prepared by hot pressing was harmless, though without promoting growth.

The successful use of cotton seed *flour* in the ration of growing chicks was referred to in a recent paper from our laboratory.[14]

[12] Cf. Osborne and Mendel, *J. Biol. Chem.*, 1913, xv, 311; Mixture IV, described on p. 317, was used.

[13] McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 361.

[14] Osborne and Mendel, *J. Biol. Chem.*, 1916, xxvi, 293.

Two of these birds (Nos. 5 and 6) beginning at the age of 28 days on a corn gluten + cotton seed flour ration gained 450 gm. (No. 5) and 556 gm. (No. 6) respectively in 79 days.

With diets containing cotton seed kernels[15] *our feeding trials have had an entirely different outcome.* All rats, both young and adult, receiving this product in the ration have promptly died within two weeks.

Here for the first time we have met with suggestions of toxicity so frequently attributed to cotton seed products. It must be noted that in the case of our cotton seed *kernel* feedings, in contrast with all other diets containing cotton seed preparations, the rats soon refused the food. This raises the question whether or not the unheated kernels contain something which causes the rats to refuse this food so that death was due to starvation rather than to any real toxicity of the cotton seed kernels. A control experiment with twelve rats (six young, six adults), deprived of all food except water, showed that these died almost as quickly as those fed with the scarcely eaten cotton seed *kernel* rations.

The statistics of the trials are given below:

*Rats on Cotton Seed Kernel Food.**

Rat.	Duration of life.	Food intake.	Original weight.	Final weight.	Loss.	
			Large rats.			
	days	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
3246 ♀	8	40.8?	174	120	54	31
3349 ♂	7	12.3	245	183	62	25
3514 ♂	7	37.9	233	180	53	23
3516 ♂	8	19.6	300	195	105	35
3518 ♂	7	55?	174	123	51	29
3576 ♂	11	37.5?	185	114	71	38
Averages.....	8	4.2 per day.	219		66	30

* The composition of the food mixture was as follows:

	per cent
Cotton seed *kernels*	66
"Protein-free milk"	28
Butter fat	6

[15] These were kindly furnished by Dr. F. E. Carruth of the North Carolina Agricultural Experiment Station.

Rats on Cotton Seed Kernel Food—Concluded.

Rat.	Duration of life.	Food intake.	Original weight.	Final weight.	Loss	
Small rats.						
	days	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
3672 ♀	5	4.7	86	58	28	33
3673 ♀	5	7.4	82	60	22	27
3676 ♀	6	10.0	86	64	22	26
3678 ♀	5	6.1	81	55	26	32
3679 ♀	6	7.8	80	54	26	33
3680 ♀	6	8.7	80	53	27	34
Averages.....	5.5	1.3 per day.	83		25	31

Rats without Food.

Rat.	Duration of life.	Original weight.	Final weight.	Loss.	
Large rats.					
	days	*gm.*	*gm.*	*gm.*	*per cent*
3462 ♀	6	173	115	58	34
3469 ♂	12	287	160	127	44
3472 ♂	7	247	150	97	39
3592 ♂	6	214	129	85	40
3600 ♂	6	180	108	27	40
3602 ♂	6	190	118	72	38
Averages.....	7	215		78	39
Small rats.					
3713 ♀	3	70	49	21	30
3717 ♂	4	79	50	29	37
3718 ♀	4	74	47	27	37
3720 ♀	3	69	46	23	33
3721 ♀	4	70	45	25	36
3722 ♀	4	83	54	29	35
Averages.....	4	74		26	35

A further illustration of the depressing effect of the presence of cotton seed kernels upon the food intake, with its untoward consequences upon body weight, is shown even in the experiments in which the *kernels* form a smaller fraction of the protein concentrate. When the protein, as measured by its nitrogen

content, was derived in approximately equal quantities from casein and cotton seed *kernels*, mixed together in the ration, death followed a prompt decline in the food intake, although on the previous casein ration the voluntary food intake had been sufficient for growth (see Chart V, Rats 3712, 3719).

Similar consequences resulted when cotton seed *flour* and cotton seed *kernels* were included in essentially equal proportions in the ration (see Chart V, Rat 3384). In the record of Rat 3509 (Chart V) it will be observed that the food intake promptly declined whenever cotton seed *kernels* were included in an otherwise satisfactory ration containing cotton seed *flour*. Even so little of the *kernels* as is represented by 2 per cent of protein produced an unmistakable effect, though this was by no means so marked as was the case when larger quantities of the kernels were present. Recovery, associated with increased food intake, occurred whenever the *kernels* were withdrawn from an otherwise (qualitatively) unchanged food mixture.

A number of experiment station workers who have investigated cotton seed meal as a food for the various types of farm animals have noticed the failure of these animals to eat as much of the cotton seed meal rations as they did of the other protein concentrates employed.[16]

[16] Cf. Armsby, H. P., and Hess, E. H., *Pennsylvania Agric. Exp. Station, Bull. 28*, 1894, 22;
Burkett, C. W., *North Carolina Agric. Exp. Station, Bull. 189*, 1903;
Curtis, R. S., *North Carolina Agric. Exp. Station, Bull. 215*, 1911;
Curtis, G. W., and Carson, J. W., *Texas Agric. Exp. Station, Bull. 21*, 1892;
Emery, F. E., and Kilgore, B. W., *North Carolina Agric. Exp. Station, Bull. 87*, 1892;
Georgeson, C. C., Burtis, F. C., and Otis, D. H., *Kansas Agric. Exp. Station, Bull. 53*, 1895;
Hartwell, B. L., and Lichtenthäler, R. A., *Rhode Island Agric. Exp. Station, Bull. 156*, 1914;
Hooper, J. J., and Anderson, W. C., *Kentucky Agric. Exp. Station, Bull. 176*, 1913;
Jeffrey, J. S., *North Carolina Agric. Exp. Station, Bull. 211*, 1910;
Lloyd, E. R., *Mississippi Agric. Exp. Station Report*, 1902, 17;
Nourse, D. O., *Virginia Agric. Exp. Station, Bull. 121*, 1901;
Smith, H. R., *Nebraska Agric. Exp. Station, Bull. 100*, 1907;
Vanderford, C. F., *Tennessee Agric. Exp. Station Bulls.*, 1893, vi, No. 2;
Wells, C. A., and Ewing, P. V., *J. Biol. Chem.*, 1916, xxvii, 15; *Georgia Agric. Exp. Station, Bull. 119*, 1916.

If our results are indicative of the presence of a deleterious factor in the cotton seed *kernels* in contrast with the *meal* or *flour*, the question arises as to the cause of the difference between these products. It might seem that some objectionable constituent is removed from the cotton seed when the oil is expressed from the kernels in the commercial manufacture of the meals. On the other hand, it may be that the *heating* of the kernels prior to the extraction of the oil facilitates destruction of any toxic component that is present. This appears to be the view of Withers and Carruth as expressed in personal communications to us. To obtain some answer to the questions thus raised the following series of experiments was conducted.

A sample of the ground cotton seed *kernels* which had been proved inadequate for promoting growth was pressed *without heating* in a hydraulic press for 24 hours at a pressure of about 2,000 pounds. The press cake thus obtained was ground and placed between successive layers of blotting paper and subjected to an additional pressure of 4,300 pounds for 4 hours to remove as much of the oil as possible. About 30 per cent was expressed by this method. A portion of the residue was then thoroughly extracted with ether, part of it being allowed to stand in a bottle under ether and shaken frequently for about 2 weeks, and the rest of it being extracted in a Soxhlet apparatus for about a week. The ether extract thus obtained after removal of the ether was a dark reddish brown fatty solid equal to about 10 per cent of the press cake taken. The residual *ether-extracted cotton seed meal* was fed to two rats and proved to be entirely adequate as the source of protein for promoting their growth at a normal rate (see Rats 3813♂ and 3815♂, page 302). Two rats which received in their food 4.5 per cent of the *ether extract* (an amount equivalent to the quantity of residual meal which had proved to be efficient) promptly declined in weight and died in 4 and 9 days respectively (see Rats 3846♀ and 3853♀, page 302). When smaller quantities of this ether extract were fed, the rate of decline was much slower, and one rat was restored by removing the extract from the diet (see Rats 3822♀ and 3823♀, page 302). Although it is extremely difficult to extract *all* of the ether-soluble substance from the cotton seed *kernels*, it is obvious that by our

procedure something was removed from them which was either actually toxic in itself or which rendered the food so unpalatable that the rats refused to eat enough of it to sustain life.

Having demonstrated, like Withers and Carruth, that the harmful substance could be removed from the kernels by extraction with ether, the question next arose whether mere pressure would be equally efficacious. A part of the *oil* obtained by pressing the cotton seed *kernels* in the cold was heated to 110°C. for 18 hours and the rest of it left unheated. When incorporated in the food of rats neither sample was detrimental (see data on page 303). When the ration included our cold process *press cake*, still containing about 9 per cent of fat, a rapid loss of weight followed just as in feeding untreated *kernels* (see Rats 3146, 3284, 3417, 3463, 3603, page 302). In order to obtain the maximum yield of oil it is the commercial practice to heat the kernels before pressing. Samples of such *meal* which we have tested have been shown by numerous experiments to be a satisfactory food for rats.

To explain these results two hypotheses are at once suggested: either the substance which renders the *kernels* injurious is insoluble in *cold* oil and can be removed only by heating; or the injurious ingredient is actually destroyed by heat. To test the former hypothesis feeding trials were conducted in which 23 per cent of the food consisted of the crude *hot pressed oil*, obtained for us directly from the mill by Dr. Carruth. Although the food was very oily, the rats ate it readily and grew at a nearly normal rate (see Rats 3816 ♀ and 3817 ♀, page 303).

This result points to the validity of the alternative hypothesis; namely, that the injurious substance is destroyed by heat. To decide this question, samples of the untreated cotton seed *kernels* were heated for varying lengths of time under different conditions, and the resulting products incorporated in the food of rats. One sample was heated in an electric oven (dry heat) for 22 hours at 110°C. and not pressed. This treatment evidently rendered the *kernels* somewhat less injurious; for the rats fed on this sample declined more slowly than those on the *unheated kernels*, though all of them died in 11 to 24 days with an average loss of body weight amounting to 21 per cent. Another sample of 1,700 gm.

*Rats on Foods Containing Parts of the (Unheated) Cotton Seed Kernels.**

Rat.	Duration of experiment.	Food intake.	Original weight.	Final weight.	Change in weight.	
A. Cotton seed kernels, cold-pressed.						
	days	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
3146 ♀	13	20.4	199	138	−61	31
3284 ♀	4	9.5	185	151	−34	18
3417 ♂	13	50±	220	150	−70	32
3463 ♀	15	85±	162	100	−62	38
3603 ♀	7	8.7	138	95	−43	31
B. Cotton seed kernels, ether-extracted.						
3813 ♂	41	295.0	60	130	+70	117
3815 ♂	41	291.0	60	130	+70	117
C. Casein + ether extract of cotton seed kernels, 4.5 per cent.						
3846 ♀	4	7.1	60	41	−19	32
3853 ♀	9	23.9	61	38	−23	38
D. Casein + ether extract of cotton seed kernels, 2.25 per cent.						
3822 ♀	17	?	70	61	− 9	13
3823 ♀	18	?	65	58	− 7	11
E. Casein + ether extract of cotton seed kernels, 1 per cent.						
3813 ♂	26	168.8	130	139	+ 9	7
3815 ♂	26	161.6	130	137	+ 7	5

* The composition of the food mixtures was as follows:

	A. *per cent*	B. *per cent*	C. *per cent*	D. *per cent*	E. *per cent*
Cotton seed *kernels*	49.5	41.5			
Ether extract of cotton seed kernels			4.5	2.25	1
Casein			18.0	18.00	18
"Protein-free milk"	28.0	28.0	28.0	28.00	28
Starch		2.5	29.0	29.00	29
Lard	4.5	10.0	2.5	4.75	6
Butter fat	18.0	18.0	18.0	18.00	18

*Rats on Foods Containing Cotton Seed Oil.**

Rat.	Duration of experiment.	Food intake.	Original weight.	Final weight.	Change in weight.	
Casein + cotton seed oil, cold-pressed.						
	days	*gm.*	*gm.*	*gm.*	*gm*	*per cent*
3697♂	28	132.9	51	95	+44	86
3698♀	28	148.5	50	94	+44	88
3702♀	56	342.6	59	123	+64	108
3708♂	56	365.7	51	139	+88	173
3709♂	28	160.6	54	102	+48	89
Casein + cotton seed oil, cold-pressed, and heated to 110° for 18 hours.						
3699♂	16	68.0	54	60	+ 6	11
3700♂	17	82.5	49	69	+20	41
3712♂	17	103.9	50	81	+31	62
3715♀	20	107.3	55	73	+18	33
3719♀	17	110.9	60	80	+20	33
Casein + cotton seed oil, crude.						
3816♀	56	350±	66	113	+47	71
3817♀	56	338.6	60	125	+65	108

* The composition of the food mixtures was as follows:

	per cent
Casein	18
"Protein-free milk"	28
Starch	25
Butter fat	6
Cotton seed oil	23

was put in a cheese-cloth bag in the container of the hydraulic press. The apparatus was carefully insulated and steam passed into the material for 5½ hours. The container and kernels were then quickly transferred to the hydraulic press and as much of the oil as possible was pressed out. The press cake, from which the oil was by no means completely removed, was dried at about 60° and fed to two rats.

Other samples of the ground *kernels* were subjected in jacketed containers to vigorous treatment with live steam for different lengths of time, from 1 to 6 hours. The kernels reached a tem-

perature of 104–109°C. There was some loss through distillation and spraying. The steamed kernels were dried at about 60°C. and ground.

The results of all of these experiments with the heated cotton seed kernels are tabulated below.

*Rats on Heated Cotton Seed Kernel Foods.**

Rat.	Duration of experiment.	Food intake.	Original weight.	Final weight.	Change in weight.	
A. Kernels, not pressed, heated for 22 hours at 110°C. in the electric oven.						
	days	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
3737♂	13	53.1—	67	49	−18	27
3738♂	20	58.2—	65	49	−16	25
3739♂	11	29.3	69	58	−11	16
3741♂	23	76.6	57	46	−11	19
3743♂	23	72.7	56	47	− 9	16
3744♂	24	79.3	60	45	−15	25
Averages.....	19	3.5 per day.	62		−13	21
B. Kernels, not pressed, steamed 1 hour.						
3723♀	12	27.9	54	41	−13	24
3724♂	18	75.2	50	47	− 3	6
3732♀	12	41.3	47	40	− 7	15
3734♂	12	33.8	45	40	− 5	11
3748♀	16	68.1	47	40	− 7	15
3750♀	14	47.7	50	40	−10	20
Averages.....	14	3.4 per day.	49		− 8	15
C. Kernels, partially pressed, steamed 5½ hours.						
3836♀	42	321.6	60	133	+73	122
3851♀	42	310.8	70	121	+51	73
Averages.....	42	7.5 per day.	65		+62	98

Rats on Heated Cotton Seed Kernel Foods.—Concluded.

Rat	Duration of experiment.	Food intake.	Original weight.	Final weight.	Change in weight.	
			D. Kernels, not pressed, steamed 2 hours.			
	days	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
3860 ♀	28	186.8	75	108	+33	44
3863 ♂	28	163.3	57	82	+25	44
3874 ♀	28	204.7	72	119	+47	65
3875 ♂	28	206.5	61	112	+51	84
3878 ♂	28	221.6	60	126	+66	110
3893 ♀	28	178.5	58	98	+40	69
Averages.....	28	6.9 per day.	64		+44	70
			E. Kernels, not pressed, steamed 4 hours.			
3861 ♀	28	209.0	75	118	+43	57
3864 ♂	28	163.4	54	89	+35	65
3876 ♂	28	203.9	66	113	+47	71
3882 ♀	28	156.7	55	92	+37	67
3888 ♂	28	192.1	55	90	+35	64
3894 ♀	28	159.4	50	81	+31	62
Averages.....	28	6.5 per day.	59		+38	64
			F. Kernels, not pressed, steamed 6 hours.			
3862 ♀	28	163.2	65	81	+16	25
3866 ♂	28	152.6	57	86	+29	51
3877 ♂	28	159.3	55	83	+28	51
3887 ♀	28	158.1	63	82	+19	30
3889 ♂	28	136.3	55	69	+14	25
3892 ♀	28	155.0	56	79	+23	41
Averages.....	28	5.5 per day.	59		+22	37

* The composition of these food mixtures was as follows:

	A. *per cent*	B. *per cent*	C. *per cent*	D. *per cent*	E. *per cent*	F. *per cent*
Cotton seed *kernels*..	61	66	52.5	60.5	56	59.5
"Protein-free milk".	28	28	28.0	21.0	21	21.0
Lard.....			1.5		5	11.5
Butter fat.....	11	6	18.0	18.5	18	18.0

These experiments show that by *steaming* the *kernels* for a sufficient length of time, they may be rendered apparently harmless for feeding. These results which correspond in many respects to the observations of Withers and Carruth, not yet published in detail, are, however, by no means uniform. In some cases *kernels* which had been heated for longer periods of time proved to be less satisfactory as food than those subjected to less prolonged steaming. It is not inconceivable that undue heating may render the meal unpalatable or otherwise unsuitable for nutrition, in addition to destroying the original deleterious substance. These facts may help to explain the conflicting evidence regarding the alleged suitability of different samples of commercial meals. The chemical reactions by which "gossypol" or any other objectionable ingredient can be altered to render it harmless have not yet been adequately investigated. Until the effects of heating cotton seed have been more fully studied, conclusive statements on this feature cannot be made.

The following tabular summary gives the statistics of average data compiled from our feeding experiments, with cotton seed products of various descriptions, relating to the source of the deleterious factor. The compositions of the food mixtures containing these products are indicated elsewhere in the text.

The facts now available may be briefly summarized as follows.

Cotton seed *kernels* are unsatisfactory for nutrition. Such samples of cotton seed *meal* and *flour* as have been tested were valuable foods for growing rats, both when used as the sole source of protein in the food, or when used in smaller quantity to supplement other less efficient protein concentrates.

The injurious substance in the *kernels* can be removed by extraction with ether and, according to Withers and Carruth, by extraction with carbon bisulfide, chloroform, benzene, or alcohol, but not with petroleum ether or gasoline. The ether-soluble material is deleterious, either because it contains some toxic ingredient or because it renders the food containing it so unpalatable that the animals refuse to eat it. This is in accord with the results obtained by Withers and Carruth, and by McCollum, Simmonds, and Pitz.[13]

Foods containing cotton seed oil prepared by pressing the kernels in the cold, or furnished as the crude unbleached com-

Experiments with Food Mixtures Containing Cotton Seed Products. Averages.

No. of rats.	Food.	Food intake per day.	Duration of experiment.	Original weight.	Change in weight.
		gm.	*days*	*gm.*	*per cent*
6	None.	0	Death in 7 days.	215	−39
6	"	0	" " 4 "	74	−35
6	Untreated kernels.	4.2	" " 8 "	219	−30
6	" "	1.3	" " 6 "	81	−31
6	Kernels heated dry at 110° 22 hours.	2.9	" " 20 "	62	−21
6	Kernels steamed 1 hour.	3.5	" " 14 "	49	−15
6	" " 2 hours.	6.9	28	64	+70
6	" " 4 "	6.5	28	59	+64
6	" " 6 "	5.5	28	59	+37
2	" " 5½ " and partly pressed.	7.5	42	65	+98
5	Kernel residues cold-pressed.	1.8	Death in 10 days.	181	−30
5	Oil cold-pressed.	5.4	28	53	+45
5	Oil cold-pressed and heated at 110° 18 hours.	6.5	17	53	+20
2	Oil, crude.	6.1	56	63	+90
2	Kernels ether-extracted.	7.2	41	60	+117
2	Ether extract of kernels, 4.5 per cent.	2.2	Death in 7 days.	61	−35
2	Ether extract of kernels, 2.25 per cent.	?	18	68	−12
2	Ether extract of kernels, 1 per cent.	6.6	26	130	+6

mercial oil prepared by heating the kernels before pressing them, are eaten without detriment by rats.

By treatment with steam under suitable conditions the *kernels* lose their deleterious effect on rats. The variations in the results of feeding different samples of cotton seed meal, which have been reported, may be due to differences in the mode of heating which the products have experienced in their preparation.

Reverting to the question already raised as to whether so called "cotton seed injury" in the feeding of domestic animals can be classed with the deficiency diseases, it is quite possible

that, as Rommel and Vedder maintain, food mixtures lacking some of the now recognized essential ingredients of an adequate diet have been employed in the past. Our experience with rats successfully grown on cotton seed rations excludes the probability that there is ordinarily any lack of the water-soluble vitamine. Whether the quota of inorganic salts furnished in agricultural practice is always sufficient we are unable to answer. It is noteworthy, however, that we have induced young rats to double their weight at a normal rate of growth on a food mixture containing nothing except cotton seed *meal*, starch, and lard. The deleterious effects of unheated cotton seed *kernels* cannot be denied. Whether the reputed detrimental effect after feeding some of the commercial cotton seed *meals* is associated with a failure to destroy a deleterious constituent—as has been indicated above—or is attributable to unsuitable methods of feeding in some cases is still debatable. The treatment of the cotton seed so as at least to render it harmless now seems to lie within the range of ready possibilities.

APPENDIX.

CHART I. Showing normal growth of rats on rations furnishing protein in the form either of the isolated *cotton seed globulin* or the *total alkali-soluble cotton seed protein.*

The food mixtures had the following composition.

	per cent	*per cent*
Cotton seed globulin	18	
Alkali-soluble cotton seed protein		18
"Protein-free milk"	28	28
Starch	28	24
Lard	8	12
Butter fat	18	18

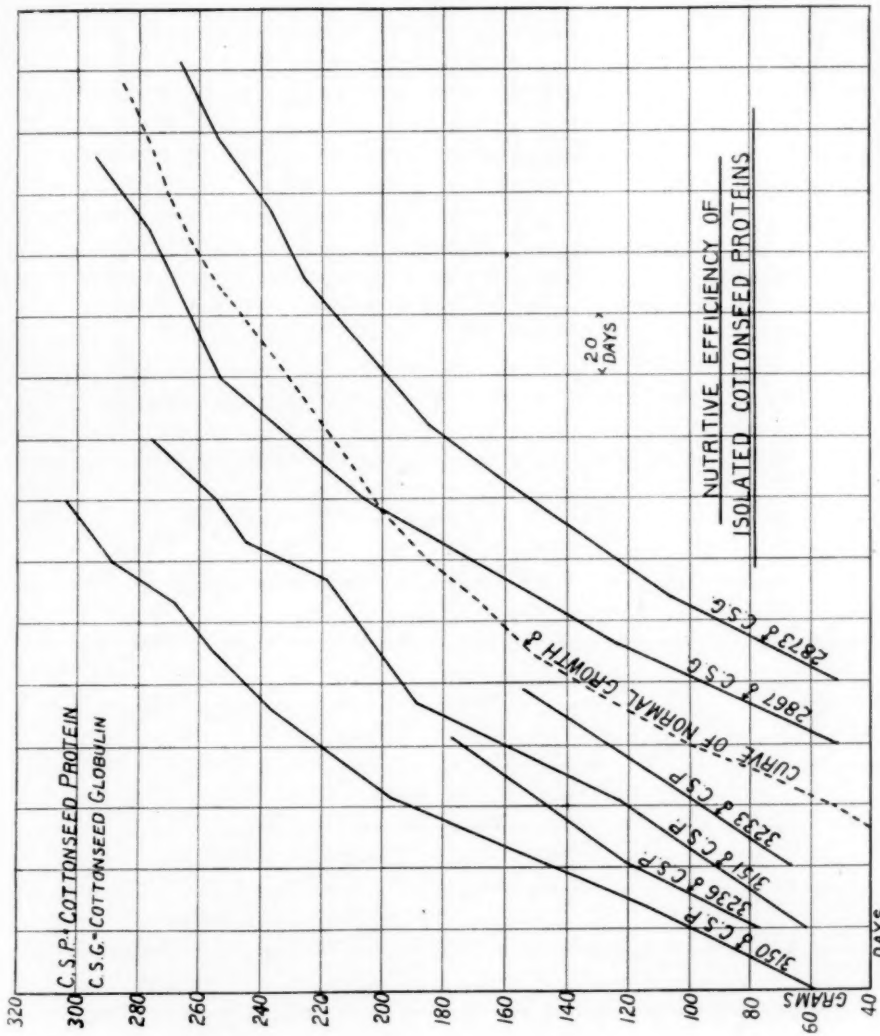
NUTRITIVE EFFICIENCY OF
ISOLATED COTTONSEED PROTEINS
C.S.P. COTTONSEED PROTEIN
C.S.G. COTTONSEED GLOBULIN
20 DAYS
CURVE OF NORMAL GROWTH
2873 & C.S.G.
2867 & C.S.G.
3233 & C.S.P.
3137 & C.S.P.
3236 & C.S.P.
3150 & C.S.P.
GRAMS
DAYS
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40

CHART I.

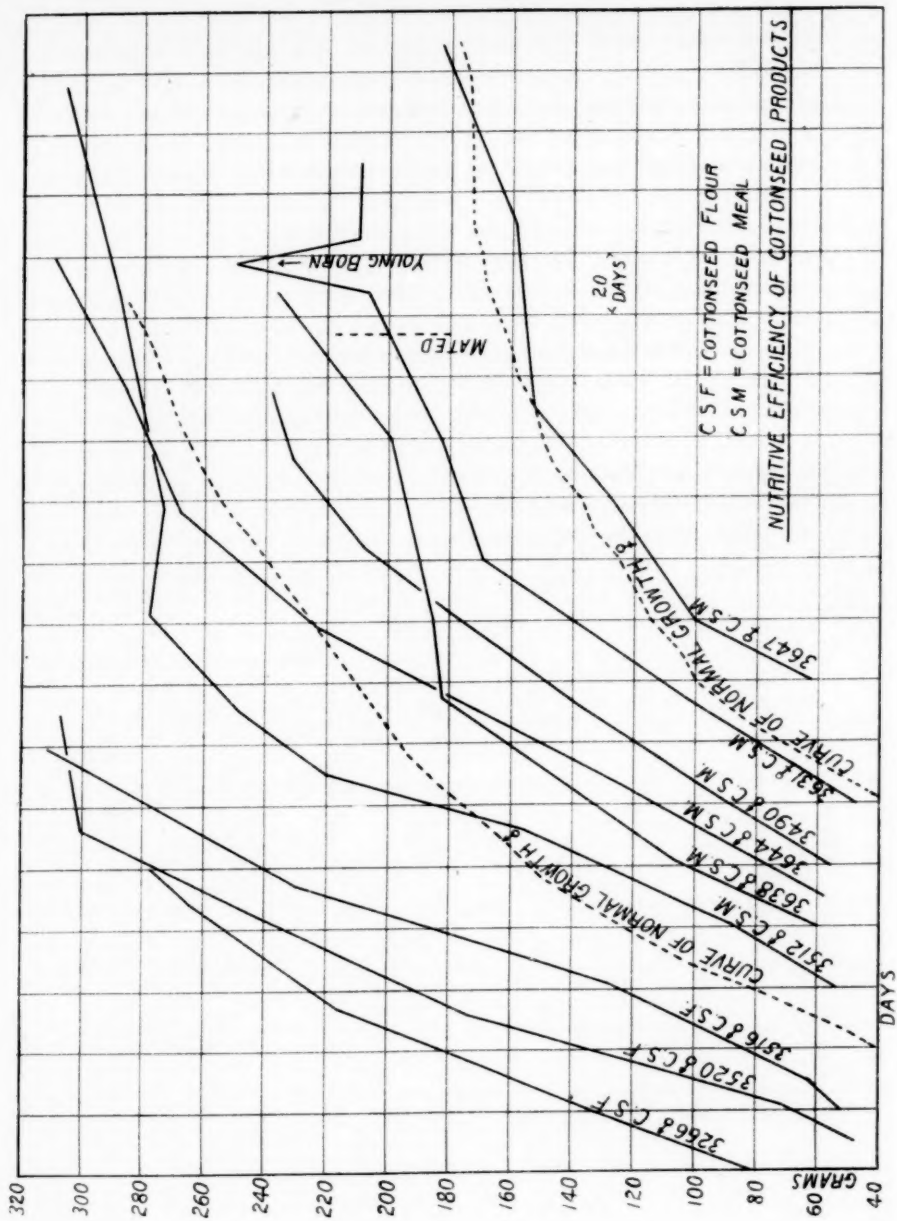
NUTRITIVE EFFICIENCY OF COTTONSEED PRODUCTS
C S F = COTTONSEED FLOUR
C S M = COTTONSEED MEAL
20 DAYS
YOUNG BORN
MATED
CURVE OF NORMAL GROWTH
3647 ♀ C S M
3631 C S M
3490 ♂ C S M
3644 ♂ C S M
3638 ♂ C S M
3512 ♂ C S M
CURVE OF NORMAL GROWTH ♂
3516 ♂ C S F
3520 ♂ C S F
3266 ♂ C S F
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
GRAMS
DAYS

CHART II. Showing the excellent growth of rats on foods in which the protein (except for the traces in the "protein-free milk") was furnished either by cotton seed *flour* or cotton seed *meal*. Suitable salts and vitamines were incorporated in the food mixtures which had the following composition.

	per cent	*per cent*
Cotton seed *flour*	41	
Cotton seed *meal*		41.0–52.5
"Protein-free milk"	28	28.0
Starch	2	0.0– 2.0
Lard	11	1.5–11.0
Butter fat	18	18.0
Approximate protein content	18	15.5–18.0

Rat 3631 ♀ mated with Rat 3644 ♂ gave birth to six young as indicated in the chart.

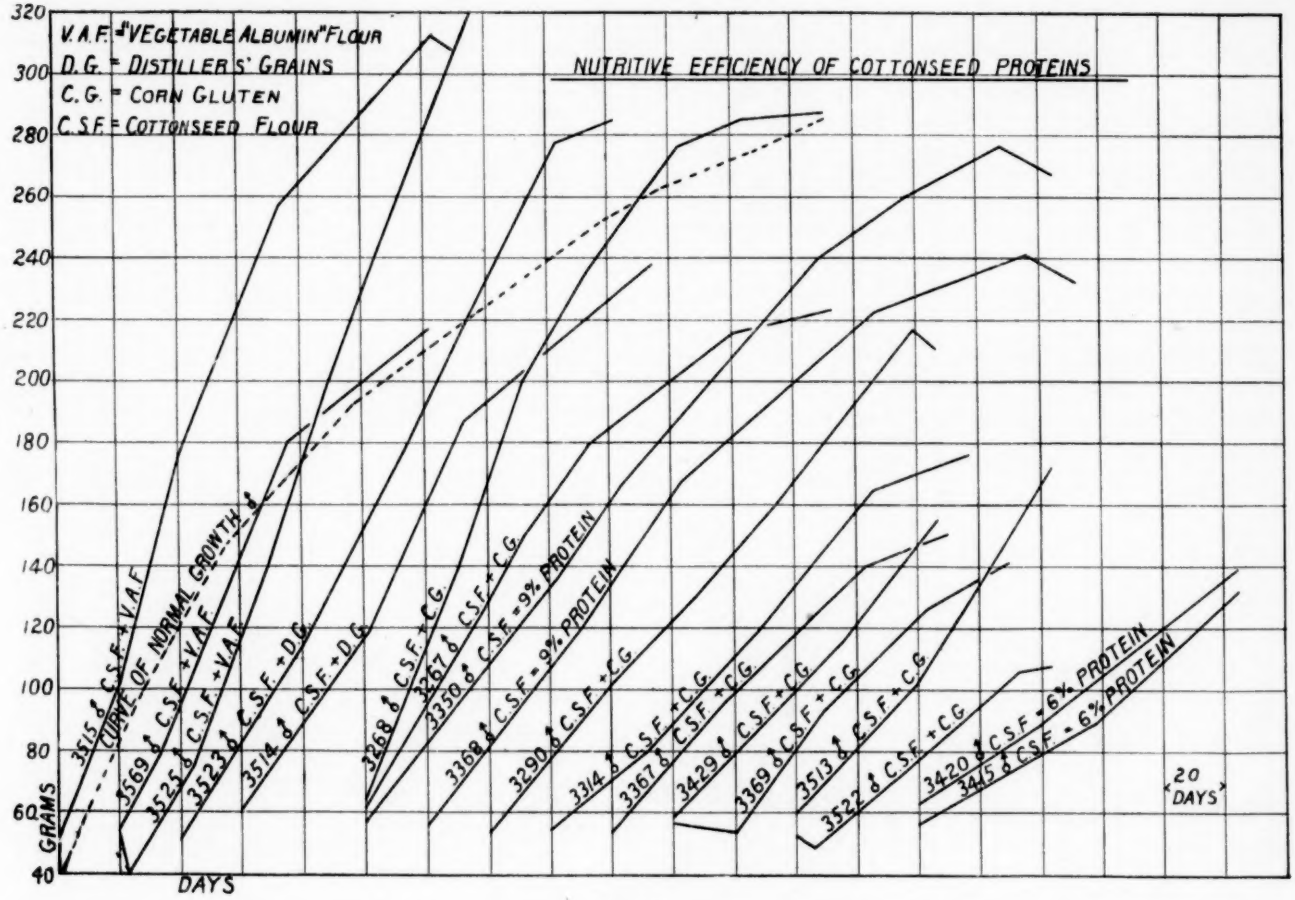
V.A.F. = "VEGETABLE ALBUMIN" FLOUR
D.G. = DISTILLER'S GRAINS
C.G. = CORN GLUTEN
C.S.F. = COTTONSEED FLOUR
NUTRITIVE EFFICIENCY OF COTTONSEED PROTEINS
CURVE OF NORMAL GROWTH
3515 ♂ C.S.F. + V.A.F.
3569 ♂ C.S.F. + V.A.F.
3525 ♂ C.S.F. + V.A.F.
3523 ♂ C.S.F. + V.A.F.
3514 ♂ C.S.F. + D.G.
3268 ♂ C.S.F. + C.G.
3267 ♂ C.S.F. + C.G.
3350 ♂ C.S.F. + C.G.
3368 ♂ C.S.F. = 9% PROTEIN
3290 ♂ C.S.F. + C.G.
3314 ♂ C.S.F. + C.G.
3367 ♂ C.S.F. + C.G.
3429 ♂ C.S.F. + C.G.
3369 ♂ C.S.F. + C.G.
3513 ♂ C.S.F. + C.G.
3522 ♂ C.S.F. + C.G.
3420 ♂ C.S.F. = 6% PROTEIN
3415 ♂ C.S.F. = 6% PROTEIN
GRAMS
DAYS
20 DAYS
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40

CHART III.

CHART III. Showing the high nutritive efficiency of cotton seed *protein* as exemplified by the excellent growth of rats upon rations containing comparatively low contents of protein in the form of cotton seed *flour* (Rats 3350, 3368, 9 per cent protein; 3415, 3420, 6 per cent protein); likewise by supplementing satisfactorily other protein concentrates—corn gluten (Rats 3267, 3268, 3290, 3314, 3367, 3369, 3429, 3513, 3522), "vegetable albumin flour" (Rats 3515, 3525, 3569), and distillers' grains (Rats 3514, 3523)—which alone failed to yield all of the amino-acids required for normal growth.

The food mixtures had the following composition.

Rats	3350, 3368.	3415, 3420.	3514, 3523.	3515, 3525, 3569.
	per cent	*per cent*	*per cent*	*per cent*
Cotton seed flour (N = 8.1 per cent)	20.5	14	14	20.5
"Vegetable albumin flour" (N = 13.1 per cent)				10.0
Distillers' grains (N = 4.7 per cent)			36	
"Protein-free milk"	28.0	28	24	28.0
Starch	27.5	30		12.5
Sucrose		4		
Lard	6.0	6	8	11.0
Butter fat	18.0	18	18	18.0

Rats	3267, 3268.	3290, 3314.	3367, 3369, 3429.	3513, 3522.
	per cent	*per cent*	*per cent*	*per cent*
Cotton seed flour (N = 8.1 per cent)	20.5	13.7	10.0	8.2
"Corn gluten" (N = 6.6 per cent)	19.0	25.3	28.5	30.4
"Protein-free milk"	28.0	28.0	28.0	28.0
Starch	3.4	6.0	6.5	7.4
Lard	11.1	9.0	9.0	8.0
Butter fat	18.0	18.0	18.0	18.0

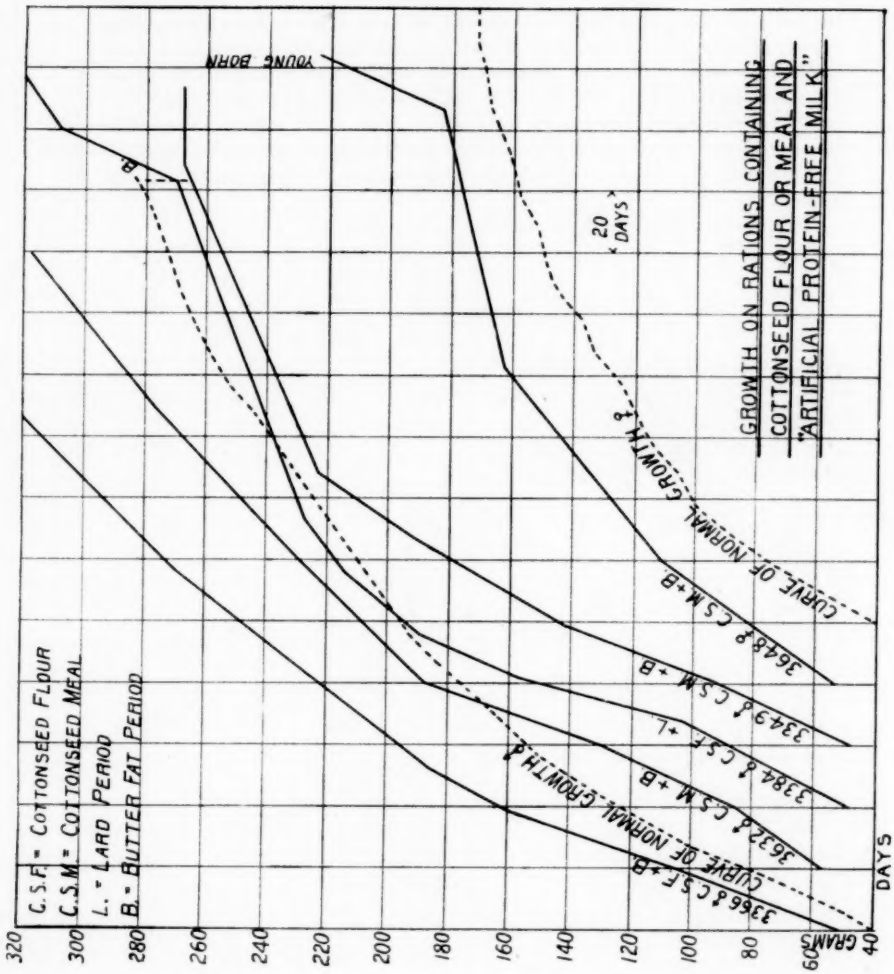
GROWTH ON RATIONS CONTAINING
COTTONSEED FLOUR OR MEAL AND
"ARTIFICIAL PROTEIN-FREE MILK"
CURVE OF NORMAL GROWTH
20 DAYS
YOUNG BORN
3366 ♂ C.S.F. +B
3632 ♂ C.S.M. +B
3384 ♂ C.S.F. +L
3349 ♂ C.S.M. +B
3648 ♂ C.S.M. +B
C.S.F. = COTTONSEED FLOUR
C.S.M. = COTTONSEED MEAL
L. = LARD PERIOD
B. = BUTTER FAT PERIOD
GRAMS
DAYS

CHART IV.

CHART IV.

CHART IV. Showing growth on rations in which cotton seed *meal* or *flour* was the source of the protein and water-soluble vitamine. The satisfactory growth resulting indicates that these dietary factors are present in suitable form in these cotton seed products.

The food mixtures consisted of:

Period	Butter fat.	Lard.	Butter fat.
	per cent	*per cent*	*per cent*
Cotton seed meal	52.5		
Cotton seed flour		41.0	41.0
"Artificial protein-free milk"	28.0	29.5	29.5
Starch		1.5	3.5– 1.5
Lard	1.5	28.0	8.0–10.0
Butter fat	18.0		18.0

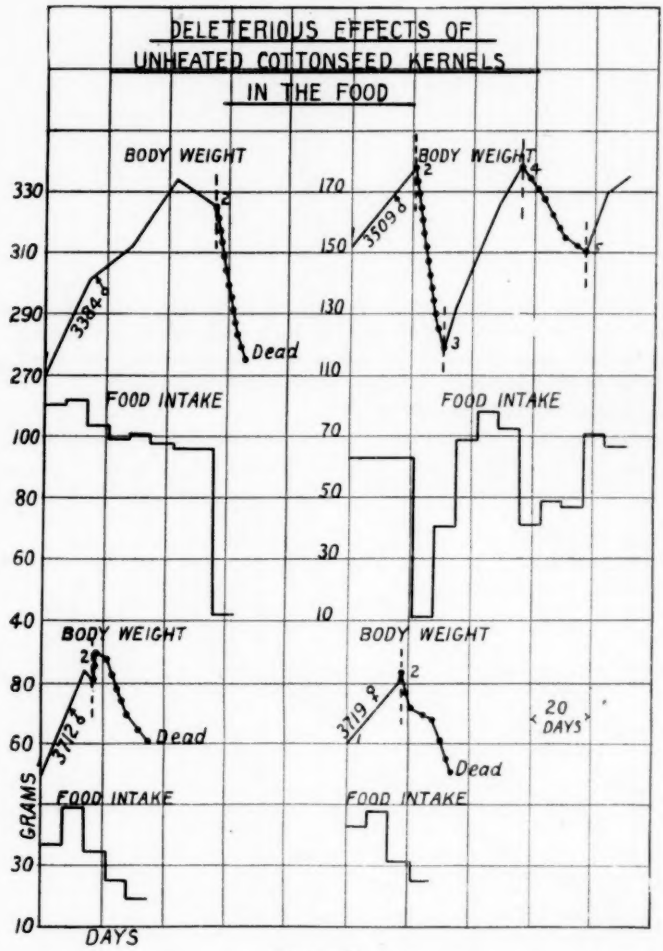
DELETERIOUS EFFECTS OF
UNHEATED COTTONSEED KERNELS
IN THE FOOD
BODY WEIGHT
FOOD INTAKE
Dead
3384 g
3509 g
3712 g
3719 g
20 DAYS
GRAMS
DAYS

CHART V.

CHART V. Showing the fall in body weight and decline in weekly food intake when cotton seed *kernels* were admixed in rations otherwise demonstrated satisfactory for growth. The periods during which the kernels were included in the food are indicated by the beaded line (-●-●-●-●-●-). The resumption of growth is shown in rats when the cotton seed *kernels* were excluded from the diet.

The food mixtures had the following composition.

Rats	3509.	3384.	3384.	3509.	3509
Periods	1, 3, and 5.	1.	2.	2.	4.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Cotton seed flour	26.3	41.0	13.2	13.2	21.7
Cotton seed kernels			33.0	33.0	7.7
"Protein-free milk"	28.0			28.0	28.0
"Artificial protein-free milk."		29.5	29.5		
Starch	21.7	3.5	9.3	10.8	18.6
Lard	6.0	8.0	3.0	3.0	6.0
Butter fat	18.0	18.0	12.0	12.0	18.0
Approximate protein content.	11.5	18.0	14.2	14.2	11.5

Rats	3712 and 3719.	
Periods	1.	2
	per cent	*per cent*
Casein	18	9.0
Cotton seed kernels		33.0
"Protein-free milk"	28	28.0
Starch	25	14.5
Cotton seed oil	23	
Lard		3.5
Butter fat	6	12.0

THE COMPOSITION OF ADIPOCERE.

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It is to Fourcroy (10), who in 1787 examined the waxy remains of the bodies of some children in Paris, that we owe the name adipocere. This waxy substance resulting from chemical change in buried animals has, under the names of *Leichenwachs, Cera cadaverica,* and Adipocere, been reported upon a number of times. Fourcroy considered it a soap of ammonia and phosphate of lime, the fats from which had a melting point of 52.5°C., and he grouped it with cholesterol and spermaceti.

Chevreul in 1812 considered a specimen of human adipocere to be a mixture of margaric and oleic acids combined with yellow odorous substances containing lime, potash, and nitrogenous matter.

Gregory (1) in 1847 made a study of hog's adipocere and concluded that it contained 75 per cent of palmitic acid, 25 per cent of stearic acid, and 2 to 3 per cent of lime. He was the first to observe the absence of glycerides, as the fat gave no acrolein reaction. The stearic acid described by him had a melting point of 79° and was obviously the mixed hydroxy stearic acids described below. He found also a small quantity of cholesterol.

Ebert (2) in 1875 found a specimen of pig's adipocere to contain about 1 per cent of ammonia and 6 per cent of insoluble residue, and the fatty acids to consist chiefly of margaric and palmitic. He found no stearic or oleic acids but recognized an acid melting at 80°C. as a hydroxy acid which he decided after analysis to be hydroxy margaric acid. This is probably the "stearic acid" of Gregory and is a mixture of two isomeric hydroxy stearic acids isolated by us from the adipocere reported in this paper.

Schmelck's (3) analyses in 1902 of three specimens, two human and one of a pig, are the only recent analyses of which we can find any record. He determined only the general physical and chemical constants of the extracted fats and fatty acids. All three gave similar analyses. Briefly his results were, melting point 62.5°C., insoluble fatty acids 83.84 per cent, ash 1.64 to 1.79, containing 84 per cent of CaO, 16 per cent of unsaponifiable matter, acid value 197, neutral value of acids 202 to 203, iodine value of acids 14.4. He made no attempt to separate the fatty acids or to study the material insoluble in ether.

Wetherill (4) some years ago, and Tarugi (5) in 1905, as the results of partial analyses, concluded that adipocere consisted chiefly of palmitic acid.

About a year ago a fine mass of adipocere was given to one of us by Mr. R. J. Blair of Ormstown, Que. It was found on a farm near Rockburn, Que., in an uncultivated wet spot, and was probably buried for 45 to 47 years. The specimen consisted chiefly of a slab 1 to 3 inches thick of the back of a pig, identified as such by some hair that remained and a portion of the jaw found with it. It weighed 15½ pounds.

The adipocere was, when cleaned from adhering earth, a hard white wax as resonant as a piece of hard paraffin, and quite homogeneous; portions taken from different parts of the mass showed almost identical physical and chemical properties. Running through the cleaned pieces fine strands of connective tissue could easily be detected.

A preliminary qualitative analysis showed that the wax was chiefly composed of ordinary saturated solid fatty acids, lime soap, fatty acids insoluble in petroleum spirit, and some connective tissue.

Quantitative Analysis.

The analyses were made on average samples taken from different parts of the mass. The finely ground material after drying *in vacuo* over sulfuric acid was first extracted in a Soxhlet with rhigolene, a very light petroleum spirit (sp. gr. 0.625, boiling point 22°C.), to separate the acids insoluble in petroleum spirit from the soluble fats and acids. The mean of a number of extractions gave 78.3 per cent soluble in rhigolene. The portion insoluble in the petroleum spirit was then extracted in a Soxhlet with ether and a further average yield of fatty acids of 15.8 per cent was obtained, leaving 5.9 per cent of insoluble residue.

The Rhigolene-Soluble Material.—This was found to be composed chiefly of palmitic with stearic and oleic acids in small quantities, together with still smaller quantities of fat and unsaponifiable matter. This mixture gave a mean melting point of 51.5–51.9°C., an acid value of 207.1, a saponification value of 210.7, or a mean molecular weight of 266.1. This last value, while it lies between those of stearic acid (284) and palmitic acid

(256), is a little high, owing to the presence of small quantities of neutral fat and unsaponifiable matter.

To obtain an idea of the quantitative relations of the constituents, we first attempted to separate the stearic acid by the Hehner and Mitchell method (6), but practically no stearic acid was obtained. It seemed from our experiments that the method is unsatisfactory and that a large excess of palmitic acid inhibits the separation of the stearic acid in alcoholic solution at 0°C. A prolonged and tedious fractional crystallization from 95 per cent alcohol was then employed to separate the acids. Six or seven series were carried through, involving a great number of separations.

The results briefly stated were: (1) The isolation of a non-saponifiable neutral substance almost insoluble in alcohol which crystallizes in rhombic spear-shaped waxy crystals giving a constant melting point of 64.5°C. This is optically inactive and gives none of the reactions of cholesterol or allied substances. The quantity obtained in a pure form (0.7 gm.) was too small to permit of accurate identification. (2) The separation of pure stearic acid to an amount corresponding to 3.3 per cent of the original adipocere. This is probably too low, as the separation of stearic acid from palmitic by fractional crystallization, or by their magnesium salts, is not accurately quantitative. (3) There was no margaric acid present. The crystals melting about 59–60°C., which occasionally formed, were eutectic mixtures of stearic and palmitic acids easily separated by varying the conditions of crystallization or by their salts into the two acids. (4) The quantity of neutral fat present, which was quite soft and pasty, was very small, only 1.37 per cent of the adipocere. This was calculated from the difference between the saponification and acid values of the mixture, and confirmed by direct extraction, after neutralization of all free fatty acids. This neutral fat gave an iodine value of 10.37, corresponding to 11.6 per cent of olein. Palmitic and oleic acids were obtained from this fat after saponification, but no stearic acid was obtained. (5) The unsaturated acids present calculated to oleic acid were 5.24 per cent of the original adipocere. These were calculated from the iodine value (Wijs' method). A liquid acid was separated from the soluble residues left from the fractional crystal-

lization, by the lead salt ether or Gusserow-Varrentrapp (7) method. Owing to the presence of some saturated acids whose lead salts were partially soluble in ether, this acid gave the low iodine value of 44.8. (6) There were only doubtful traces of volatile fatty acids.

Fatty Acids, Etc., Obtained by Ether Extraction of Adipocere.

This mixture was a solid waxy mass and contained all the fatty acids uncombined as calcium soaps together with the small quantities of fats and unsaponifiable matter. This wax gave the following results, which were determined for comparison with the constants obtained by Schmelck:

Melting point	60–63°C.
Acid value	201.7
Saponification	207
Mean mol. wt	288
Acetyl value	34.75

The higher melting point and the lower acid and saponification values of this mixture compared with the extract by rhigolene show clearly the presence of acids of higher molecular weight.

The refractive index determined by the Abbé Zeiss refractometer was 1.4395 at 65°C.

Hydroxy Stearic Acids.

The hard waxy mixture making up the fraction insoluble in rhigolene but soluble in ether and constituting 15.8 per cent of the entire adipocere gave a melting point of 71°C. After partial purification the crystals obtained melted at 78–79°C., corresponding to the stearic acid of Gregory and to the oxy margaric acid of Ebert. They gave an acid value of 183.35, corresponding to a molecular weight of 306. (The molecular weight of hydroxy stearic acid is 300.) These crystals consisted of mixed rhombic plates and long quadratic prisms.

On careful fractional crystallization from hot ether, two well defined acids were separated, the more insoluble separating from hot ether at room temperature in prisms, often 2 cm. long, with oblique terminal planes. These when purified by recrystal-

lization melted sharply at 84.5°C. From the mother liquid by cooling to 0°C. another acid with an entirely different habit of crystallization was obtained in small glistening rhombic plates melting at 77–78°C., which on recrystallization finally melted at 78.5°C. These two acids were present in the proportion of about three of the former (melting point 84.5°C.) to two of the latter (melting point 78.5°C.).

On acetylation they proved to be monohydroxy acids. The acetyl value was obtained with difficulty and acetylation was incomplete even when boiled with acetic anhydride in presence of anhydrous sodium acetate for several hours. The acetyl value obtained was 157.2. The theoretical value (rarely attained experimentally) of monohydroxy stearic acid is 164. The filtration method described by Lewkowitsch (8) was employed. The acetyl value shows, however, that the acids were monohydroxy acids, and the molecular weight of each acid calculated from its acid value was 306, which points to monohydroxy stearic acid (mol. wt. 300) and not to hydroxy margaric (mol. wt. 284). Their silver salts gave on analysis 25.93 per cent and 25.97 per cent of silver. The theory for monohydroxy stearic acid was 26.53 per cent, and for hydroxy margaric acid 27.41 per cent.

In its general and physical properties the acid melting at 84.5° seems to be identical with a hydroxy stearic acid prepared from oleic and the so called "isooleic acid." It corresponds with the ι-hydroxy stearic acid (produced by treating a solution of oleic acid in petroleum spirit with sulfuric acid), whose constitution was established by Shukoff and Schestakoff (9) in 1903. This acid gave a refractive index of 1.439 at 90°C. Its silver salt crystallized in groups of short spear-shaped crystals. At 21°C. 100 gm. of absolute ether dissolved 2.02 gm. of the acid. 100 gm. of absolute alcohol dissolved 1.87 gm. at 0°C., and 9.17 gm. at 20.7°C. Its alcoholic solution was optically inactive.

The other monohydroxy stearic acid crystallizing in rhombic plates and melting at 78.5°C. is not described in the literature and may be peculiar to adipocere. This acid gave a refractive index of 1.443 at 81°C. or 1.440 when calculated to 90°C., practically identical with the ι acid (1.439). Its silver salt crystallized in long silky needles. It is much more soluble in both absolute ether and alcohol than its isomer; 100 gm. of absolute ether

dissolved 3.48 gm. of the acid at 21°C., and 100 gm. of absolute alcohol dissolved 4.41 gm. at 0°C., and 17.70 gm. at 20.7°C. Its alcoholic solution showed no optical activity.

The position of the hydroxyl group in the molecule of this acid has not yet been determined. If we assume that these acids are derived from oleic acid, it seems highly probable that it is the hitherto undescribed θ-hydroxy stearic acid. It has been satisfactorily established that the double linking in the molecule of oleic acid occurs between the θ and ι carbon atoms; *i.e.*, in the middle of the molecule. Unless we allow the possibility of another oleic acid in pig's fat with double linking in a different place, it seems unlikely that the hydroxyl group could be introduced elsewhere in the acid molecule than on the θ or ι carbon atoms.

The constitution of the acid melting at 84.5°C. has been proved by Shukoff and Schestakoff to be the ι-hydroxy stearic acid; it seems probable, therefore, that its isomer has the hydroxyl attached to the other carbon atom with double linking; *viz.*, the θ atom.

The chemical relation of these acids to oleic acid is evident from the following formulas.

Oleic acid $CH_3(CH_2)_7CH = CH(CH_2)_7COOH$.

ι-Hydroxy stearic acid $CH_3(CH_2)_7\underset{OH}{CH}\,CH_2(CH_2)_7COOH$.

θ-Hydroxy stearic acid $CH_3(CH_2)_7CH_2\underset{H}{\overset{OH}{C}}(CH_2)_7COOH$.

These two waxy acids then represent a certain portion of the fluid oleic acid, resulting from hydrolysis of olein, which has become fixed through the assumption of a molecule of water by each molecule of the unsaturated oleic acid. As stated above, the mixture of these acids was noticed in adipocere by Gregory in 1847 and called stearic acid, and was also described by Ebert in 1875 under the name of oxy margaric acid. They are probably characteristic of all adipocere.

That neither of these acids was present in its lactone form was satisfactorily established. The saponification and acid values of each acid were identical.

The Residue Insoluble in Ether.

The analysis of the residue insoluble in ether gave the following percentage composition.

	per cent
Calcium, palmitate, and stearate	61.04
Calcium hydroxy stearate	13.76
Total calcium soaps	74.8
Excess CaO	0.94
Protein	11.27
Fe_2O_3	0.48
MgO	0.24
Silica, etc	1.14
Humus and undetermined	11.13

The total ash was 0.578 per cent calculated to the original adipocere. The calcium soaps were decomposed by hydrochloric acid and the dry residue containing the acids was extracted with rhigolene as before, followed by a second extraction using ether as a solvent. By this method after recrystallization it was found that 12.94 per cent of the total residue consisted of hydroxy acids and 57.2 per cent of mixed stearic and palmitic acids. The acids soluble in rhigolene gave a melting point of 65–66°C.; and an acid value of 197.2, corresponding to a mean molecular weight of 284, showed them to consist largely of stearic acid. The hydroxy acid when recrystallized gave a constant melting point of 77–78°C. and a molecular weight of 303.3 calculated from its acid value. This is the θ-hydroxy stearic acid; no prismatic crystals of the more insoluble ι isomer could be separated.

The Nitrogenous Constituents of Adipocere.

The ammonia was determined by distilling about 10 gm. in ammonia-free water with excess of alkali. The mean of three determinations which were fairly concordant gave 0.0351 per cent calculated to the original adipocere or 0.0289 per cent of N as ammonia. The total nitrogen in the adipocere was determined by the Kjeldahl method and the mean of three analyses gave 0.1778 per cent. The nitrogen from the insoluble residue was 0.1074 per cent and was calculated to protein.

These results may be tabulated thus:

	per cent
N as ammonia	0.0289
N as protein	0.1074
N soluble (by difference)	0.0415
Total N	0.1778

The above result of the study of the nitrogenous constituents of adipocere is opposed to the view that adipocere contains an appreciable quantity of ammonium soaps. The total nitrogen is chiefly derived from the fine threads of dry connective tissue embedded in the waxy mass.

SUMMARY AND CONCLUSIONS.

In this communication an attempt has been made to obtain a quantitative analysis of mature adipocere. While the results are not of the same order of accuracy that one expects in an inorganic analysis, they are as nearly exact as our known methods of separation permit (Table I).

TABLE I.

Percentage Composition of Hard Clean Adipocere Wax.

Palmitic acid	67.52
Stearic acid	3.3
Oleic acid	5.24
ι-Hydroxy stearic acid	9.48
θ-Hydroxy stearic acid	6.32
Stearin and palmitin	1.21
Olein	0.16
Unsaponified matter	0.87
Calcium soaps	4.41
Protein	0.665
Ash	0.578
Humus and undetermined	0.247

The fatty acids, traces of fats, etc., soluble in ether, constituted 94.1 per cent of the adipocere and gave the following physical and chemical constants.

TABLE II.

Physical and Chemical Constituents of the Ether-Soluble Matter.

Sp. gr. at 100°C	0.8436
Refractive index at 65°C	1.436
Melting point	60–63°C.
Acid value	201.7
Saponification value	207.0
Iodine value	6.04
Acetyl value	34.75

The total nitrogen present was only 0.1778 per cent, of which only 0.0289 per cent was ammonia nitrogen.

As a result of the studies recorded in this paper we are justified in concluding that adipocere is the residue of the preexisting fats of animals. It is composed almost entirely of the insoluble saturated fatty acids left after the slow hydrolysis of the fats in wet ground. The protein matter has entirely disappeared and the glycerol, soaps, etc., resulting from the hydrolysis have been carried away in aqueous solution.

The insoluble hydroxy stearic acids which are so characteristic of adipocere are probably derived from a portion of the oleic acid in the original fat by hydration.

No margaric acid or oxy margaric acid is present. Ammonia and other soluble soaps are absent; calcium soaps alone are present in small quantity in a matured specimen of adipocere.

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APPLICATIONS OF A NEW REAGENT FOR THE SEPARATION OF AMMONIA.

I. THE COLORIMETRIC DETERMINATION OF AMMONIA IN URINE.

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The direct Nesslerization methods published some time ago from this laboratory[1] were believed to meet all ordinary requirements as to speed and accuracy in the determination of ammonia, urea, etc., and it was hoped that they might remain for some time the final form for the determination of nitrogen by Nesslerization. Last fall it was found, however, that the method for the determination of ammonia cannot be made generally available for the reason that one of the necessary reagents, Merck's purified blood charcoal, is not made in this country and is not now to be obtained at any price. No other charcoal can be used, because none other abstracts creatinine. After having failed in our endeavors to find or to prepare a suitable animal charcoal having the properties of Merck's, we have been compelled to turn to other fields in our endeavor to find a suitable substitute. In the course of this search we have found a product which, though different in its action, meets our need even better than Merck's charcoal. We have accordingly modified the microchemical Nesslerization method for ammonia on the basis of this reagent.

The new product which we now use is a synthetic mineral, an "aluminate silicate," a zeolite, discovered by Gans.[2] It possesses in a superlative degree the peculiar absorptive properties characteristic of some natural zeolites, and is manufactured and used

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473. 497

[2] Gans, R., *Jahrb. k. preuss. Geol. Landesanstalt*, 1905, xxvi, 179; 1906, xxvii, 63.

on a large scale for the "softening" of water and for other industrial purposes. The crude product is sold under the trade name "Permutit."

In the early part of our investigation we used a sample of permutit which had been in our possession for several years. This product had been imported from Germany. In order to be sure that we were not again working with a product which could not be made generally available we communicated with the makers of the American permutit and obtained samples of their product.

The two products were not quite identical in their properties, and by the means at our disposal we were unable to make them identical. The makers of the product solved this part of our problem for us, and by a modified process have made a reagent which is satisfactory for our purposes.

The essential mechanical feature of this new reagent for absorbing ammonia is that it is a clean, moderately fine, insoluble powder which gives off no dust or turbid material to water, and settles, like sea sand, from water in the course of a few seconds. By virtue of this novel feature the (absorbed) ammonia can be separated by decantation from the solution (or urine) which contained it.

The removal of ammonia by this mineral reagent is not an adsorptive phenomenon. The reagent is a complex insoluble sodium salt containing active, *i.e.*, easily replaceable, sodium, and the absorption of ammonia involves the replacement of a part of this sodium by ammonia. The chemical affinity of the active group in the reagent for ammonia is remarkably strong so that under suitable conditions the exchange becomes quantitative as far as the ammonia is concerned. The reaction is a reversible one, however, and in the presence of more than small amounts of soluble sodium salts, or other electrolytes, the equilibrium reached does not represent a quantitative absorption of the ammonia. 1 gm. of the powder takes up about 13 mg. of ammonia nitrogen in the course of a few minutes' gentle agitation and in the presence of slight excess of ammonium salt (15 mg. N). Yet 2 gm. of the reagent will not quantitatively remove 1 mg. of ammonia nitrogen in the presence of 100 mg. of sodium chloride. In the presence of 20 to 25 mg. of sodium chloride the removal

of 1 mg. of ammonia nitrogen is complete and 0.5 mg. will be removed quantitatively from as much as 50 mg. of salt. Notwithstanding the balanced character of the reaction, there is therefore an adequate margin of safety when working with urine, provided that one does not attempt to work with too much ammonia.

While the chemical reaction involved in the absorption of ammonia by this reagent is apparently a reaction between a solid and a solution, it remains to be said that the solid powder contains about 20 per cent of water, and if this water of hydration is removed by heat the activity of the reagent is lost. Even gentle dry heat (100°C.) greatly reduces its activity, so that a freshly purified and rapidly dried product is less active than the same product allowed to dry at ordinary temperatures, or than the same product dried rapidly at 100°C., and allowed to "weather" for a day or two.

Ammonia is absorbed best from neutral solutions, but is also absorbed well from weakly acid solutions. The presence of much acid is not good because the reagent is dissolved by acids. In alkaline solutions ammonia is not absorbed. Indeed, the serviceability of the reagent depends fundamentally on the fact that in the presence of sodium hydroxide the absorbed ammonia is again set free. The speed with which the absorbed ammonia is liberated depends very much on the quality of the reagent and also, of course, on the fineness of the powder. Some of the samples investigated held the ammonia so firmly that we did not secure its quantitative liberation except by using hot water (60–65°). As we found that ammonia can be Nesslerized at such temperatures we thought at one time that we had better incorporate the use of warm water in our description of the method; but we have not wished to do so, partly because the use of warm water is less convenient, and partly because in less experienced hands the use of warm water would almost certainly lead to occasional breakage of the colorimeter.[3]

[3] The product is made by The Permutit Company, 30 East 42nd Street, New York. We understand that the Company is prepared to furnish the powder in purified form and in any desired degree of fineness. We recommend the powder which passes through a 60 mesh sieve, but does not pass through an 80 mesh sieve. For other purposes a somewhat coarser powder may prove more useful.

One remarkable feature of those reagents which show a strong tendency to retain a part of the absorbed ammonia is that the longer the ammonia has remained in the absorbed condition the more difficult it is to get it all out. While we believe that no reagent will be sold for use according to our method, which possesses an unusual tendency to retain the ammonia after the addition of alkali, we suggest that every one purchasing a supply of the reagent should determine for himself how long the Nesslerized mixture must stand to develop the maximum amount of color. With our reagent not less than 95 per cent of the theoretical value is obtained in 2 to 3 minutes, and substantially theoretical figures are obtained in 10 to 15 minutes, or less. If the absorbed ammonia has been left in the powder over night the liberation of the ammonia takes a little longer and a deficit of 2 to 3 per cent may remain.

An important characteristic of this reagent for the absorption of ammonia is that it does not appreciably deteriorate by being used. After washing away the Nesslerized ammonia and surplus alkali first with water, then with one portion of 2 per cent acetic acid, then once more with water, the powder remaining is just as efficient as before for the absorption of more ammonia. We have made as many as eight successive quantitative determinations of 1 mg. of ammonia with the same 2 gm. of powder, and at the end the powder was apparently in just as good condition as at the beginning.

The above mentioned repetitions were made from the standpoint of curiosity rather than for the purpose of saving the reagent. In practice it is not worth while to restore single 2 gm. charges of the powder. We either throw the powder away (though naturally not in the sink) or, after a brief rinsing, pour it into a cylinder. When so collected the powder should be digested with warm (60°C.) 1 per cent sodium hydroxide for about an hour to remove ammonia absorbed from the tap water. After rinsing away the ammonia and most of the alkali, it is treated with 2 per cent acetic acid till no longer alkaline to phenolphthalein, and after further washing with water and drying, the powder is again ready for use.

The process for the colorimetric determination of ammonia in urine by the help of the synthetic zeolite powder is as follows:

Transfer about 2 gm. of the powder to a 200 cc. volumetric flask. Add about 5 cc. of water (no more), and with an Ostwald

pipette introduce 1 or 2 cc. of urine, or with a 5 cc. pipette introduce 5 cc. of previously diluted urine (corresponding to 1 or 2 cc. of the original urine). With urines extraordinarily poor in ammonia it may be necessary to use more urine (5 cc.), but, in so far as it is practicable, it is better not to use more than 2 cc. and to employ a weaker standard (0.5 mg. of ammonia nitrogen) for the color comparison. Our reason for not wishing to use more than 2 cc. of urine is based partly on practical experience and partly on the recognition of the fact that the salts in the urine tend to prevent the ammonia absorption from being quantitative. Rinse down the added urine by means of a little water (1 to 5 cc.), and shake gently but continuously for 5 minutes. Rinse the powder to the bottom of the flask by the addition of water (25 to 40 cc.) and decant. Add water once more and decant. (In the case of urines rich in bile it is advisable to wash once or twice more.) Add a little water to the powder, introduce 5 cc. of 10 per cent sodium hydroxide, mix, then add more water until the flask is about three-fourths full. Shake for a few seconds and then add 10 cc. of Nessler's reagent, prepared according to the directions of Folin and Denis.[4] Mix, and let stand for 10 minutes or as much longer as may be convenient. Fill up to the mark with water, mix, and compare in the colorimeter with the standard. The standard should be 1 mg. or, less frequently, 0.5 mg. of ammonia nitrogen, Nesslerized in a similar manner; that is, with 5 cc. of 10 per cent sodium hydroxide and 10 cc. of the Nessler solution, and the whole diluted to a volume of 200 cc.

It will be noted that in the above Nesslerization process we use only 10 cc. of Nessler's reagent, whereas Folin and Denis in the direct Nesslerization method employ 15 cc. We have introduced this slight change largely for reasons of economy. It is not necessary to add as much as 5 cc. of 10 per cent sodium hydroxide, to set free the absorbed ammonia—1 cc. would do equally well. But 10 per cent sodium hydroxide is less costly than the Nessler reagent, and by using 5 cc. of the former 10 cc. of the latter become adequate.

If the addition of alkali and Nessler's reagent has been done carelessly so that the reagent strikes the bottom of the flask while the powder still contains either too much ammonia not yet liberated, or too much of the

[4] Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 479, 480.

added alkali, the powder will turn reddish from the precipitated mercury-ammonia compound. This color can be almost instantly removed by the addition of a little 2 per cent potassium iodide solution, when washing the powder for the next determination.

It will be observed that in the dilution of the Nesslerized ammonia we take no account of the presence of about 2 gm. of the insoluble powder remaining in the flask. Theoretically this may introduce an error of something less than 1 per cent. Practically this is not so, however, because the concentration of ammonia remaining in the powder is greater than the concentration of ammonia in the Nesslerized supernatant solution. The process is also simplified by thus leaving the powder in the flask.

In the table are given some analytical results demonstrating the accuracy of this new procedure. The colorimetric determinations were made exactly as described above. The check determinations were made by Folin's macro aeration process, and

Ammonia Nitrogen in Urine per Liter.

No.	Urine.	New colorimetric method.			Aeration method. NH_3 N
		Urine used.	Colorimetric reading, standard at 20 mm.	NH_3 N.	
		cc.		*gm.*	*gm*
1	Diabetic.	2	20.1	0.50	0.50
2	Much bile.	1	21.3	0.94	0.95
3	Strong $FeCl_3$ reaction.	2	17.0	0.30	0.30
4	Albumin.	2	14.5	0.69	0.71
5	Diabetic.	2	19.8	0.25	0.25
6	Strong $FeCl_3$ reaction.	2	21.4	0.47	0.47
7	" " "	2	19.2	0.26	0.26
8	Bile, trace.	1	17.4	1.15	1.19
9	Albumin.	2	27.4	0.18	0.19
10	Diabetic.	2	19.2	0.52	0.52
11	"	2	22.0	0.46	0.45
12	"	2	15.4	0.32	0.33
13	Albumin.	2	17.9	0.56	0.54
14	Bile, albumin.	1	17.8	1.12	1.10
15	Diabetic.	2	23.5	0.43	0.42
16	Albumin.	2	25.0	0.40	0.39
17	Cat urine.	0.25	25.0	1.60	1.54

titration. These latter determinations were made in duplicate and were accepted as the standard only after duplicate titrations agreed to within 0.1 cc. of 0.1 N alkali. The colorimetric determinations were not made in duplicate, and it might be added that not a single repetition was required. They were made and recorded by one of us while the other was making the aeration determinations, and of course were finished first. All the urines except the last one were pathological specimens obtained fresh from the Massachusetts General Hospital.

From time to time attempts have been made in this laboratory to make use of ordinary tap water instead of distilled water in connection with the Nesslerization methods for the determination of nitrogen. These attempts have invariably failed, because when ordinary city water is used the Nesslerized solutions become turbid. The cause of the turbidity is the presence of magnesium salts in the water. Calcium salts do not interfere unless present in amounts sufficient to yield insoluble calcium carbonate. These disturbing impurities, as well as any traces of ammonia which may be present, are readily removed by agitating the water, either by shaking or by means of an air current, in the presence of a few gm. of purified permutit powder which we use for the quantitative absorption of ammonia. The impurities are likewise removed simply by filtering the water through a layer of the powder.

THE CALCIUM CONTENT OF CEREBROSPINAL FLUID, PARTICULARLY IN TABES DORSALIS.

By JOHN O. HALVERSON and OLAF BERGEIM.

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(Received for publication, February 1, 1917.)

In spite of the great number of researches which have been carried out to determine the composition of cerebrospinal fluid in health and disease, hardly anything has been known with regard to the content of calcium in this fluid either in normal or pathological conditions. Of the five single determinations that we have run across in the literature three represent analyses of fetal fluid, two of these being from hydrocephalus, a fourth is from a hydrocephalic adult, and a fifth represents mixed adult spinal fluid. Thus Schmidt (1) found 15.3 mg. of calcium per 100 cc. in a hydrocephalic adult, and 7.0 mg. in a hydrocephalic fetus. Yvon (2) found in the fetus 8.0 mg. of calcium, while Mestrezat (3) in a case of hydrocephalus found at autopsy 6.2 mg. The latter author also analyzed a mixture of twenty normal spinal fluids and found 7.1 mg. of calcium. All of these results represent more or less incidental determinations of this element and do not give us accurate information as to calcium content of the spinal fluid of adults during life. After death the composition of cerebrospinal fluid has been shown to alter so considerably as not to be comparable with the composition during life (4).

In view of the known importance of calcium for certain nervous mechanisms, the authors thought it worth while to determine whether any significant alteration of the calcium content of the spinal fluid could be noted in some of the more common brain and cord affections. It was also thought that the calcium content of spinal fluid as compared with that of the blood plasma would be of interest in connection with the origin and function of the spinal fluid.

In former analyses of spinal fluid large amounts of the liquid have been employed and the determination was therefore laborious. Using the method elaborated by the present authors (5) for the determination of calcium in blood and other body fluids accurate determinations can readily be carried out on from 5 to 10 cc. of spinal fluid, amounts which may be readily obtained during life. On account of its low protein content, spinal fluid is readily ashed for the determination and this procedure was used in most cases, but the deproteinization procedure is even here much more convenient and gives consistent results, within 2 per cent of ashing. Only one-fifth of the amount of picric acid used for the blood determination is needed in this case. 10 cc. of spinal fluid in a 25 cc. volumetric flask are treated with 10 cc. of water and 1 cc. of 4 per cent sodium picrate solution, and then 1 cc. of concentrated hydrochloric acid is added slowly with rotation. The flask is then made up to mark, heated in a boiling water bath for 15 minutes, cooled, filtered through high grade ashless paper, and an aliquot (usually 20 cc.) used for the determination by the permanganate titration of precipitated oxalate.

The average calcium content of the cerebrospinal fluids studied was 5.1 mg. per 100 cc. Most of the figures lie between 5.0 and 5.3 mg. The average for the cases of tabes dorsalis was 5.0. A value of 5.0 was obtained in the case of a normal man and similar values were obtained in other cases where the spinal fluid appeared normal. Only one specimen showed a variation of more than 0.3 mg. from this value. This was a marked case of paresis with a strongly positive Wassermann reaction, and very high globulin and cell content. The case showed 6.0 mg. of calcium per 100 cc., this value being confirmed by repeating the examination. We have found in several cases of syphilis that the calcium content of the blood plasma or serum is normal as to calcium content. Our data indicate also that repeated drainage of fluid from the spinal canal has little effect upon its content of calcium, although this has been claimed to increase the cryoscopic value (6).

In only one case were we able to obtain a calcium determination on spinal fluid of a patient with low blood calcium. This was in a case of uremic coma which showed a plasma value of 8.2 mg. of calcium per 100 cc., and in the spinal fluid 5.3 mg. There

was no parallel change in the composition of the spinal fluid in this respect such as has been observed for urea (7).

Calcium Content of Cerebrospinal Fluids.

No.	Sex.	Notes.	Calcium per 100 cc.	Wassermann.		Cells.	Globulin.
				Blood.	Spinal fluid.		
			mg.				
1	♂	Normal man.	5.0	−	−	N*	N
2	♂	Vasomotor ataxia.	5.0	−	−	N	N
3	♀	Uremia.	5.3	−	−	N	N
4	♂	Tabes dorsalis.	5.2		+	84	+
5		1 week later.	5.1		+		
6		3 weeks "	5.2		+		
7		4 " "	5.1		+		
8	♂	Tabes dorsalis.	4.8		+	N	N
9	♂	" "	5.2		+4	N	N
10		4 weeks later.	5.3		+2	33	+
11	♂	Tabes dorsalis.	5.1		+	44	+
12		4 weeks later.	5.0		−	4	N
13	♂	Tabes early.	5.2		+	24	+
14	♂	" dorsalis.	4.9		+	N	N
15	♂	" and paresis.	4.8		+	N	N
16	♀	" dorsalis.	5.3	+3	+2	N	N
17	♂	"	5.3		−	33	+
18	♂	Paresis.	5.1		+		
19	♂	"	5.3		+	44	+
20	♂	"	5.2	+	+4	38	+
21	♂	"	6.0	+	+4	187	++
22	♀	Ophthalmoplegia.	5.0	−	−	44	+
23	♂	Luetic hemiplegia.	5.0		2	N	N
24	♂	Amylotrophic, lateral sclerosis.	5.3	+4		N	N
25	♂	Brain abscess.	5.0	−	−		
26		Mixed sample, ashing method.	5.04				
27		Mixed Sample 26, deproteinization method.	5.01				
28			5.00				
29			5.03				

*Normal.

The calcium content of the cerebrospinal fluid thus tends to remain constant under the conditions studied. We have found this to be true for the blood under a considerable variety of patho-

logical conditions. This same constancy has been shown for the blood of cows during pregnancy and lactation (8). The calcium content of cerebrospinal fluid is approximately one-half that of the blood plasma or serum, normal values for which lie between 9 and 11 mg. per 100 cc. This marked difference in composition is probably in part due to the very low protein content of cerebrospinal fluid and also to the more alkaline reaction of this fluid. It has been found (9) that the cerebrospinal fluid showed a P_H of 8.11 as compared with an average of 7.66 for the blood of the same series of individuals. These authors also found no alteration in the reaction of the blood or cerebrospinal fluid in cases similar to those here reported by us.

CONCLUSIONS.

The calcium content of the cerebrospinal fluid of adults is very constant at about 5.0 mg. of the element in 100 cc. of fluid. This is approximately one-half the calcium content of the blood plasma or serum of similar individuals. The calcium content was determined in a number of cases of syphilis of the nervous system. Variation greater than 0.3 mg. from the value given was obtained in but a single very severe case. In tabes dorsalis an average value of 5.1 mg. was obtained. Repeated drainage did not appear to affect the composition of the fluid in respect to calcium.

The authors are indebted to Dr. Philip B. Hawk for the opportunity of carrying out this work, and to Drs. William H. Spencer and George P. Meyer for assistance.

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THE NATURE OF THE DIETARY DEFICIENCIES OF THE OAT KERNEL.*

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(Received for publication, January 31, 1917.)

In order to attain optimum results in human nutrition and animal production, we must have a thorough knowledge of the supplementary dietary relationships among our naturally occurring foodstuffs. Such knowledge can be gained only through the interpretation of the results of properly planned feeding experiments, since laboratory methods for the examination of foods are not sufficiently refined to do more than point out where lie the profitable lines of study by biological methods. We have acquired in our experiments with purified foodstuffs, with and without the addition of water or alcoholic extracts of various natural foodstuffs, and with fats from various sources, what we believe to be an adequate working hypothesis as to the factors which operate in making a successful diet.[1] We have proceeded to examine individually the dietary properties of the more important natural foods as revealed by feeding them with single or multiple additions of isolated dietary components. These studies have been described elsewhere for rice,[2] wheat,[3] wheat germ,[4] and maize kernel.[5]

* Published with the permission of the Director of the Wisconsin Experiment Station.

[1] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181, 231. McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916, xxv, 105; *Am. J. Physiol.*, 1916, xli, 333.

[2] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 181.

[3] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 615. Hart, E. B., and McCollum, E. V., *ibid.*, 1914, xix, 373. Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid.*, 1916, xxv, 239. McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 333.

[4] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916, xxv, 105.

[5] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916–17, xxviii, 153.

The knowledge thus acquired enabled us to proceed to the more complex problems relating to the degree to which combinations of two natural foods in various proportions supplement the deficiencies of each other.[6] In the present paper we present data showing the supplementary relations between the oat kernel and isolated food factors. The results may be briefly summarized as follows:

1. The oat kernel seems to contain proteins of a poorer quality than either the maize[5] or wheat kernel.[3] 9 per cent of oat proteins serve, when all the other dietary factors are properly adjusted, to induce a small amount of growth in the beginning of the experiment, but cessation of growth always follows after about a month and thereafter the animals remain stationary in weight or decline (Chart 6).

2. Casein does not appear to supplement the proteins of the oat kernel in a very satisfactory manner. The addition of 10 per cent of casein to 9 per cent of oat proteins, the other dietary factors being properly adjusted, does not induce growth at the maximum rate as do similar combinations of casein with wheat proteins[3] or with maize proteins.[5] Combinations of gelatin and oat proteins in about equal proportions have proven in our experience uniformly vastly superior to the similar casein and oat protein combinations.

3. The unidentified dietary factor fat-soluble A is present in very small amount in the oat kernel. It is not possible to supplement the oat kernel with inorganic salts and purified protein so as to induce growth beyond the 3rd month (Chart 3). The inclusion of butter fat or some other substance which supplies the unknown A prevents the failure at this point (Chart 5), just as it does in experiments where the ration consists of purified protein, salts, carbohydrate, and an extract which furnishes the dietary factor B.

4. The whole oat kernel, with the hulls removed in the laboratory by coarse grinding and fanning, will not induce any growth in young rats. A mixture of hulled oats or rolled oats with 5 per cent of butter fat induces very slow increase in body weight for at least 125 days (Chart 1, Lot 500).

[6] McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 333.

5. The oat kernel, like unpolished rice, wheat, wheat germ, maize kernel, alfalfa leaves, cabbage, and clover leaves, contains a liberal supply of the water-soluble B, the preparations of which induce relief from polyneuritis. This dietary factor we have found in abundance in all the natural foodstuffs in a fresh condition, so far as we have employed them in experimental work.

6. The addition of any single dietary component as protein, inorganic salts, or fat-soluble A does not supplement the oat kernel so as to induce appreciable growth (Charts 1 and 2).

7. The addition of two dietary factors to the oat kernel serves to induce good growth during the first 60 days when one of the additions is a suitable salt mixture. Without modifying the inorganic content of the ration when this is derived solely from the oat kernel, we have not seen rats make any marked increase in body weight. When the oat kernel is fed supplemented by but two dietary factors there is always early failure with loss of weight and death following the brief period of growth (Charts 3 and 4).

Failure has in our experience supervened earlier than when wheat or maize is fed with the addition of two purified food additions. The oat kernel, like the wheat kernel,[3] appears to cause injury to the animals when their diet is of such a character as to lower their vitality (Charts 2, 3, and 4). It is not necessary to assume the presence of something toxic in the oat kernel to account for the injury which results from the presence of a high content of oats in a monotonous food mixture taken over a considerable period. Oats produce feces of a pasty character which makes their elimination difficult, and in all probability tend to debilitate the animal.

This explanation becomes the more plausible when we consider the marked improvement of rats whose rations were identical except that the proteins of the oat were in one case supplemented by 10 per cent of casein and in the other by 9 per cent of gelatin. There can be no doubt that gelatin furnishes an amino-acid mixture which makes good the deficiencies of the oat proteins in a much greater degree than does casein (compare Charts 5, 8, and 9). The improved physiological condition of the animals which results from the superior quality of their protein mixture in the former case (Chart 9) may render them capable of over-

coming the difficulty of elimination of feces. If this is the real explanation, the stunting observed on the oat-casein combinations may be due to the absorption of the products of putrefaction from the intestine. Further experiments designed to test the validity of these assumptions are now under way, but several months must elapse before their records can be safely interpreted.

8. When the oat kernel is supplemented with casein, a suitable salt mixture, and butter fat, growth may proceed to the normal adult size at the normal rate in some animals, but in general growth is slower than the normal rate (Charts 5 and 7). We have been able to secure reproduction with these rations in but a single instance, and the young survived but 1 day.

Further investigations relating to the dietary properties of the oat kernel are in progress.

In all the experiments reported in this paper we have followed our usual practice of keeping from four to six rats together in a woven wire cage, the entire floor of which was covered by a pan of galvanized iron. Wood shavings were employed to keep the animals from contact with metal, and distilled water only was furnished. Iodine was supplied in the drinking water once each week in the form of iodine dissolved in potassium iodide. We also have fairly satisfactory records showing the quantitative food intake of the animals used in these experiments.

Details of the construction of the cages and of the devices used for feeding and furnishing water will be described elsewhere in the near future.

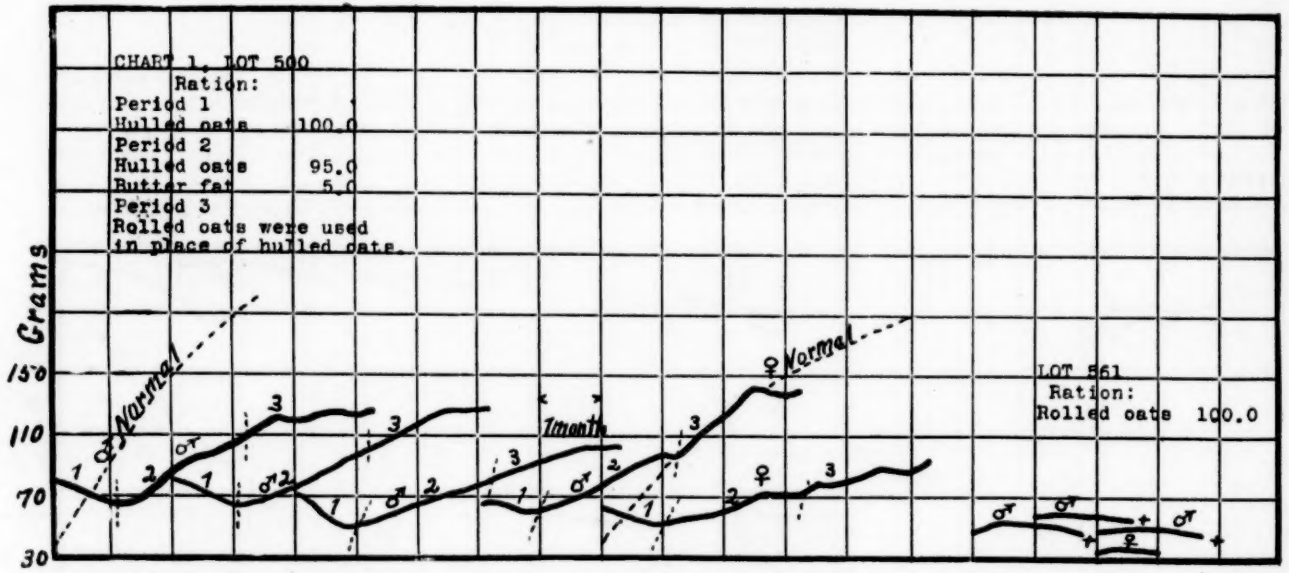

CHART 1. Lot 500 shows failure to grow and early decline in body weight of rats fed exclusively on hulled oats (Period 1) and slow but sustained growth over a period of 4 months on a mixture of hulled oats 95 and butter fat 5 per cent (Period 2). In Period 3 rolled oats were substituted for the hulled oats prepared in the laboratory, but this made no difference in the character of the curves.

Lot 561 shows the records of rats restricted to rolled oats as the sole food. Of the four animals in this group one was eaten by its companions after 4 weeks. The remaining three died at the end of the 6th and 7th weeks without having increased in body weight.

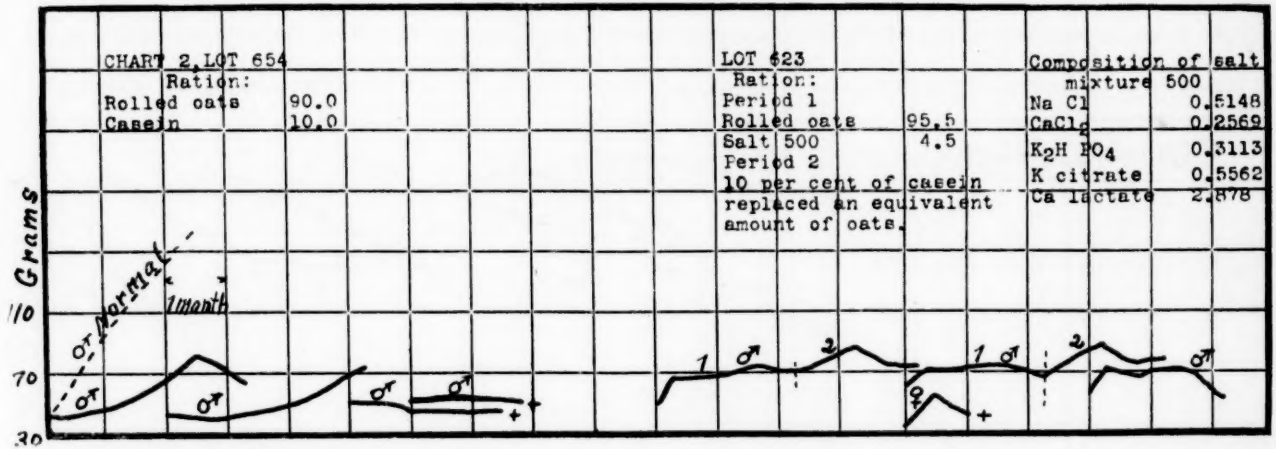

CHART 2. Lot 654 shows that young rats are unable to grow to any appreciable extent on the oat kernel supplemented with a pure protein. Two other dietary factors, the fat-soluble A, which is practically absent from oats, and the character and amount of the inorganic content must be taken into account in making good the deficiencies of the oat kernel.

Lot 623 illustrates the failure of rats to grow when restricted to rolled oats with inorganic salt additions (Period 1). The condition of the animals was such that the replacing of 10 per cent of oats by casein at the end of the 11th week did not cause growth.

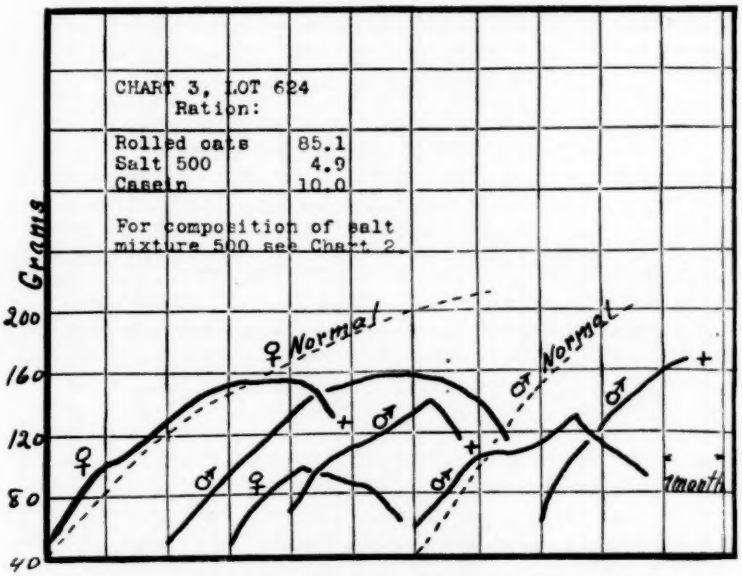

CHART 3. Lot 624 again emphasizes the marked tendency of rats to grow and apparently thrive for a brief period on rolled oats with two dietary additions; *viz.*, inorganic salts and purified casein. The rapid decline in this lot would have been entirely avoided by the inclusion in the diet of the dietary factor, the fat-soluble A, in the form of butter fat (see Chart 5). All dietary factors except the latter are contained in this food mixture and we interpret the cause of failure of this lot to the shortage of the fat-soluble A together with an injurious effect of the oat kernel on the animals.

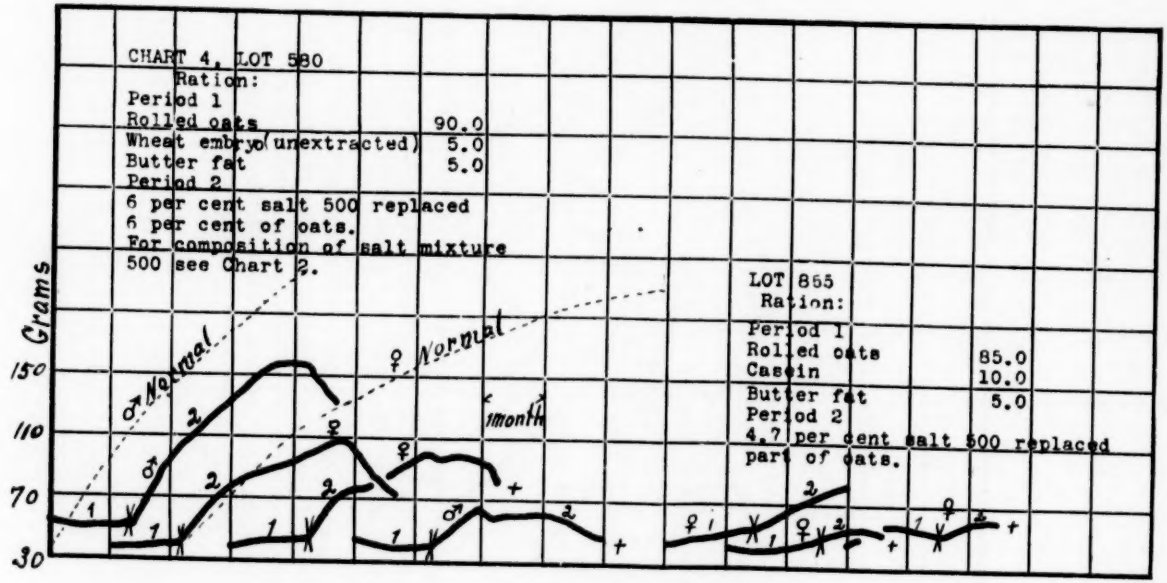
CHART 4. LOT 580
Ration:
Period 1
Rolled oats 90.0
Wheat embryo (unextracted) 5.0
Butter fat 5.0
Period 2
6 per cent salt 500 replaced
6 per cent of oats.
For composition of salt mixture
500 see Chart 2.
LOT 855
Ration:
Period 1
Rolled oats 85.0
Casein 10.0
Butter fat 5.0
Period 2
4.7 per cent salt 500 replaced
part of oats.
Grams
150
110
70
30
♂ Normal
♀ Normal
1 month

CHART 4.

CHART 4. Lot 580. This ration supplies rolled oats supplemented with the fat-soluble A in butter fat, and with an additional liberal amount of the water-soluble B in the 5 per cent of wheat embryo. In experiments with purified food mixtures 2 per cent of wheat embryo supplies enough of this factor to support growth at the normal rate.[1] 5 per cent of wheat embryo further supplies 1.5 per cent of protein of excellent quality.[4] The total protein content of the ration was 15 per cent. Even when thus supplemented rolled oats cannot support growth. After 6 weeks of growth suspension, the addition of 6 per cent of a salt mixture of suitable composition led to prompt growth at more than half the normal rate, but early failure ensued. We interpret this as supporting the evidence that the oat kernel when fed liberally causes injury to animals. Its effects are not markedly apparent when the diet is suitably adjusted as respects all its factors (Charts 5, 7, and 9) but serious injury may result when the ration is faulty for any reason, so that the vitality of the animal is lowered. In this group the vigor of the rats was impaired by the somewhat low content and poor quality of the proteins of the diet, and the debilitating effects of the oats became apparent. With a reduction in the oat content and an addition of 8.6 per cent of casein this diet is so modified that decline in early life is avoided (Chart 7). A similar diet containing oat proteins and gelatin is still more satisfactory (Chart 9).

Lot 855 illustrates the failure of rats to thrive on a diet of rolled oats, casein, and butter fat. When considered in connection with the other experiments reported in this paper it is evident that the character of the inorganic content of the ration, all of which except the phosphorus contained in the casein is furnished by the oat kernel, is the factor which is responsible for the results obtained with this food mixture (compare Charts 5, 7, and 9). The failure of these animals to respond with growth on the addition of a salt mixture after 6 weeks of stunting indicates lowered vitality.

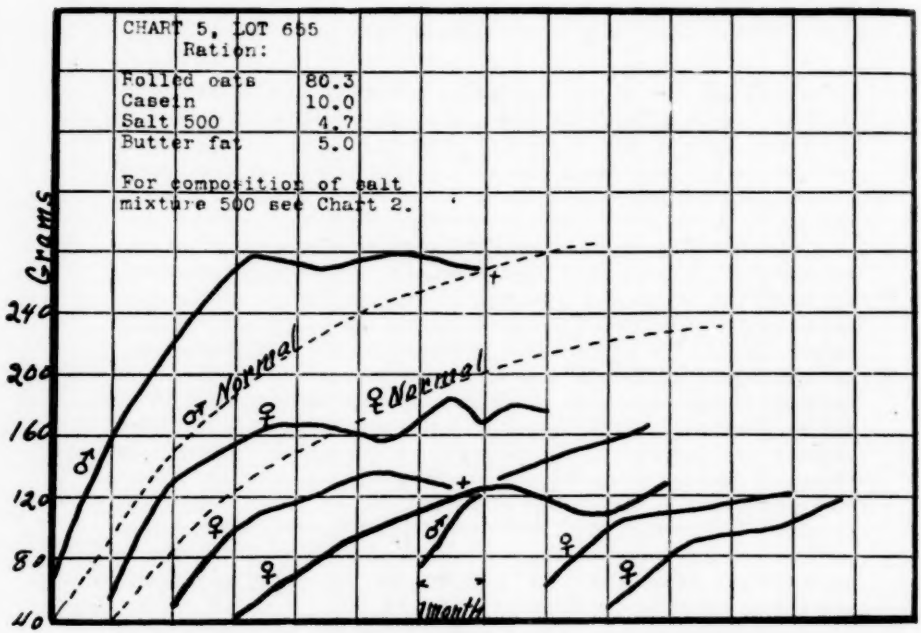

CHART 5. Lot 655 illustrates the ability of rats to grow with 80 per cent of rolled oats in their food mixture, when this is supplemented with casein, salts, and butter fat, and escape early decline and death. Without the salt mixture this diet does not support appreciable growth (Chart 4, Lot 855). Without the casein a brief period of growth is followed by decline and death (Chart 6, Lot 625). Without the butter fat (fat-soluble A) the same result is observed (Chart 3, Lot 624). Notwithstanding the presence of all essential chemical factors in this ration no reproduction was secured from these rats. This fact further supports the theory that oats exert an injurious effect when fed liberally over a prolonged period. It appears, however, from a comparison of Charts 5, 7, and 9 that casein and oat proteins do not form a protein mixture of as great value as do gelatin and oat proteins.

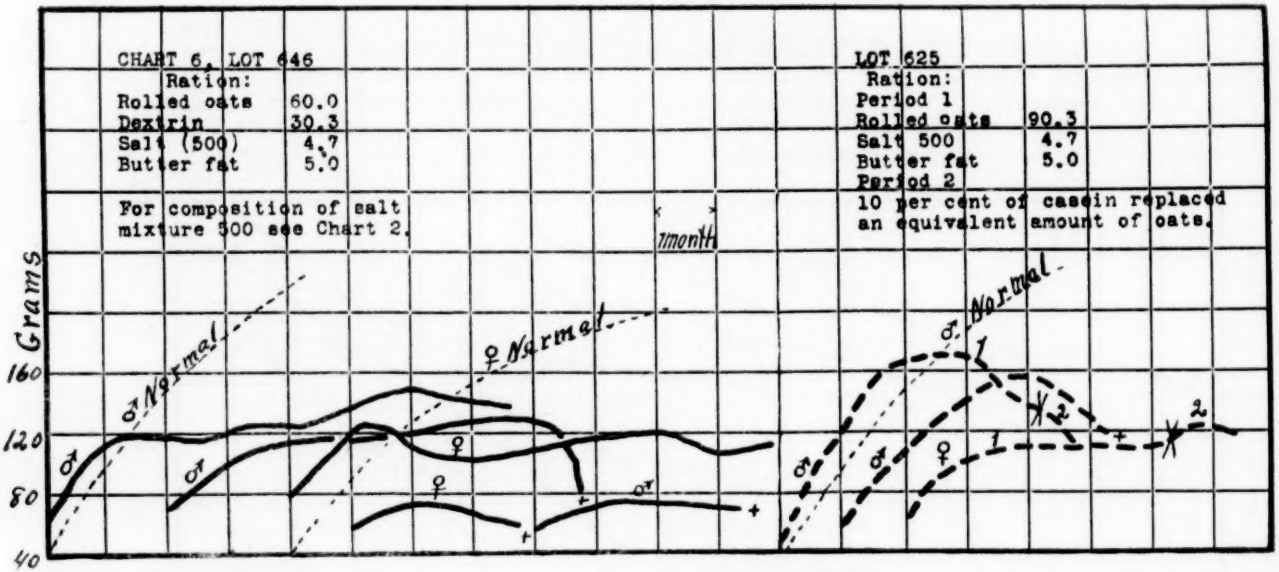

CHART 6. Lot 646 shows that 9 per cent of protein from rolled oats does not support growth beyond a limited extent. That the quality and quantity of the protein are the determining factors is made clear by comparison of these rats with those of Lot 655 (Chart 5) and with Lot 625 (Chart 6). The latter could grow but little on a similar diet containing about 13.5 per cent of oat protein, while Lot 655 (Chart 5), whose ration differed essentially from that of Lot 646 only in containing 10 per cent of casein replacing an equivalent amount of dextrin, were able in some cases to reach nearly the normal adult size.

Lot 625 illustrates the behavior of young rats given rolled oats with two additions, the fat-soluble A as butter fat and an inorganic salt addition. During 60 days growth was normal in two of the animals, but these suffered rapid decline during the 4th month. After being suspended in growth on this diet for a time the inclusion of casein in the diet did not induce recovery. The mixture of oats, salts, butter fat, and casein fed from the beginning would have promoted growth in many animals to the normal adult size without early failure (Chart 5). This ration furnished about 3.5 per cent of protein (Period 1) which is higher than is essential for the maintenance of growth[1, 7] when proteins are of good quality.

[7] McCollum and Davis, *J. Biol. Chem.*, 1915, xx, 415.

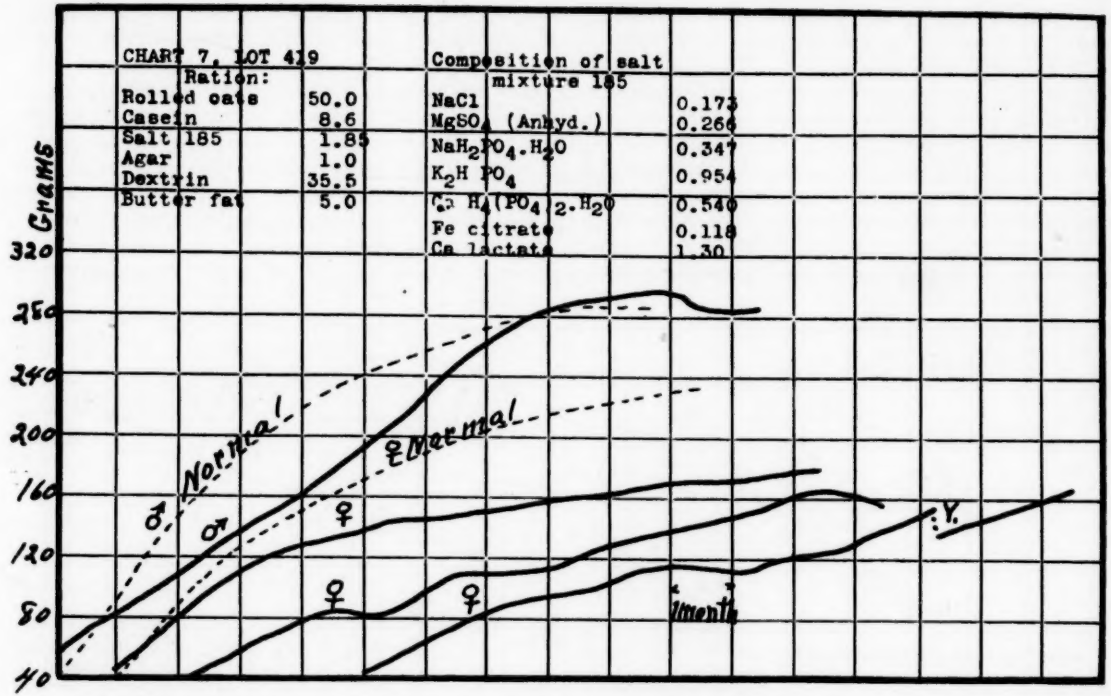

CHART 7. Lot 419 shows that it is not possible by the addition of 8.6 per cent of casein, a salt mixture, butter fat, and dextrin to 50 per cent of rolled oats, to induce the optimum of well-being in rats. Growth was not greatly depressed in the more vigorous animals, but two females during 11 months on this diet failed to produce any young, and a third produced at the age of 10 months a single litter which was immediately allowed to die. These animals were distinctly below the normal in vigor (compare with Lot 647, Chart 9).

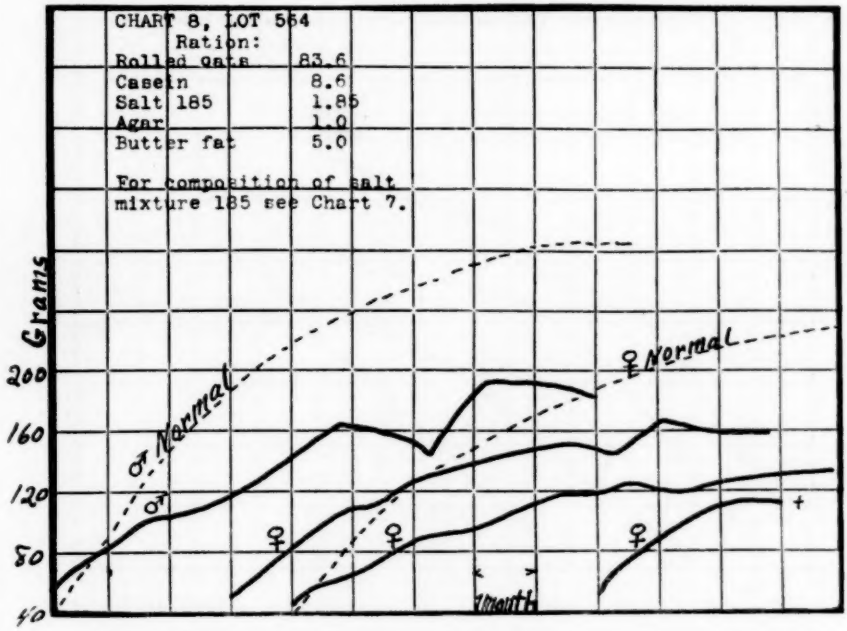

CHART 8. Lot 564 shows the records of rats whose ration was closely similar to that of Lot 419, Chart 7, except that the dextrin was replaced by oats. These rats were not quite so well nourished as were those receiving the lower oat content in the diet.

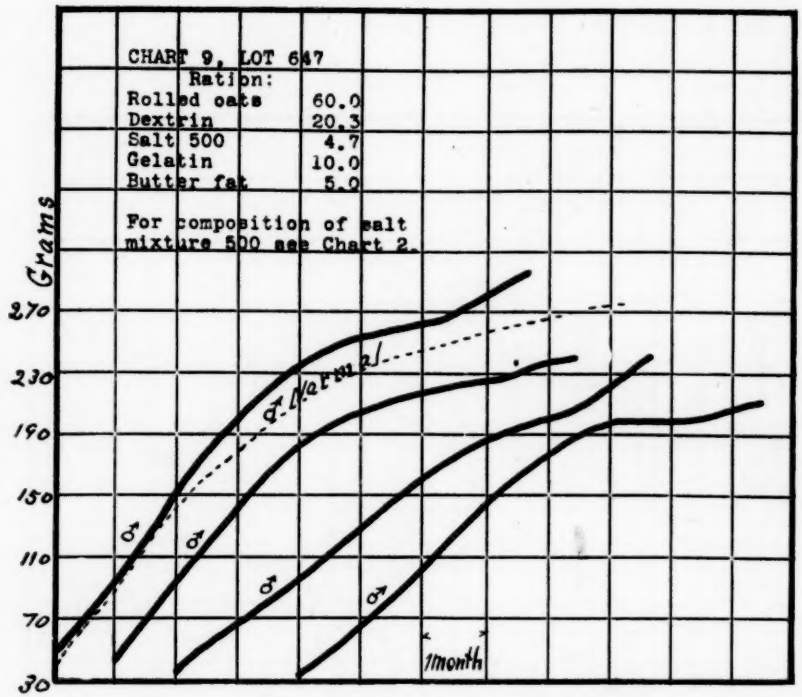

CHART 9. Lot 647 reveals the surprising fact that gelatin combined with oat proteins forms a decidedly better protein mixture than do casein and oat proteins (compare Charts 5 and 9). We have elsewhere shown that gelatin supplements well the proteins of the wheat kernel, but not at all those of the maize kernel.[8] Gelatin when taken as the sole source of protein or combined with maize is worthless. Properly combined with proteins of poor quality from certain sources it has a high biological value.

[8] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916–17, xxviii, 483.

THE METHOD OF TIMED INTRAVENOUS INJECTIONS.

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PLATES 2 AND 3.

(Received for publication, February 1, 1917.)

INTRODUCTION.

If a soluble diffusible substance, such as a sugar, an amino-acid, or a salt, is made to enter the living organism at a constant rate for a long enough time, the tendency is toward the establishment of an equilibrium which is manifested by a constancy of the rate at which the organism rids itself of the substance as such, by excretion, or chemical change, or both. The rate of entry is then equalled by the rate of utilization plus the rate of elimination.

Since, during the maintenance of a uniform entry rate, the velocity of excretion cannot vary independently without affecting the quantity of substance left over in the body for utilization and *vice versa*, the rate of utilization cannot vary independently without affecting the quantity left for excretion, and since it is known that the rates of utilization and excretion are influenced by the amount of available substance, so the occurrence either of a constant rate of elimination or a constant rate of metabolism may be used as an index of equilibrium. Now if a substance of the character mentioned is made to enter the body of a higher animal or man at a constant rate, A, until equilibrium is established, observations may then be made on the rates at which various metabolic or other physiologic processes proceed during the period of equilibrium. If then under parallel conditions the same substance is introduced at a second rate, B, bearing to A some known numerical relationship, a second equilibrium may be established with a corresponding set of observations, and so on. In this way relatively exact quantitative data may be ob-

tained of a type which should favor the interpretation of more biological processes in terms of quantitative chemistry. We have in mind especially dissociation and the laws of chemical equilibrium. In a similar way we may compare the effects produced by bringing two different substances separately into the body at the same rate, or find the rates at which two or more substances have to be given in order to produce an identical arbitrarily selected effect.

Rates and Concentration.—To establish an equilibrium during the entrance of a given substance into a given animal at a given rate naturally implies the creation of some constant concentration of the substance in the body as a whole, *provided, however, the volume of the body is kept constant*—a condition which is not fulfilled, for instance, when in spite of the establishment of an equilibrium for one substance such as glucose, there is no equilibrium with respect to another such as water. What absolute concentration is actually produced must depend on many variables, such as the character and state of the substance, the species of animal, its physiological condition, the physicochemical state of its tissues, etc. This absolute value could only be found by direct experiment for any specific case. To establish a constant concentration in the body as a whole would also imply, of course, a tendency toward the establishment of constant concentrations in each of the several tissues or body media corresponding to the relative solubilities of the substance in each, in accordance with laws which pertain to the partition of dissolved diffusoids among the phases of a heterogeneous system. The absolute values of these could also be found, if at all, only by direct observation in specific cases. *But for the purposes outlined above it is not necessary to know what concentrations are attained either in the body as a whole or in any of its parts,* although under conditions otherwise the same it might be assumed that they would vary in proportion to the rates at which the substances entered the body. In the case of simpler marine forms it is a common practice to control the rate at which an experimental substance enters the organism *by fixing the concentration of the substance in the circumambient liquid.* If the latter is changed frequently enough, or if its volume is made sufficiently great in proportion to the mass of the cells, then once the latter have ap-

proached equilibrium with the environment, molecules of the experimental substance migrate into the organism at a rate fixed by the concentration. With a gas, the concentration of which in the water is proportional to its volume percentage in the atmosphere over the water, the rate at which the substance enters the organism may be controlled by fixing its concentration in the air. And this method is available in the case of higher animals when the experimental substance is volatile like chloroform and ether, or a gas like oxygen or carbon dioxide, for the sea water then has its counterpart in the blood. The application of these principles in respiration studies by numerous workers has already led to notable achievements. *But in the case of higher animals and non-volatile substances regulation of the entry rate through the expedient merely of fixing a concentration is impractical, and recourse must be had to direct control of the entry rate into the blood, leaving the concentrations to adjust themselves.*

For well known reasons it is not possible to administer a substance by alimentary, subcutaneous, or other indirect routes and directly control the rates at which it will be taken up into the blood. On the other hand, if it is injected directly into a vein, the mechanical difficulty arises of sustaining the injection at a uniform rate for a long enough time. The problem of controlling the rate at which non-volatile substances are brought into the bodies of higher animals then resolves itself into one of mechanical apparatus.

Requirements for Apparatus.

A satisfactory apparatus should embody as many as possible of the following specifications. *In general*, it should be an instrument of precision, compact, portable, quiet in operation, for use by the bedside or in the laboratory. *The range of possible delivery rates* should be so wide as to meet the needs of experiments in laboratory animals and man. *The rate control* should be convenient and rapid, permitting of coarse and fine adjustments through a working range during the progress of a single experiment without interruption, and should allow a ready adaptation of the apparatus to the requirements of different experiments in which the ranges of rate differ widely. *An automatic rate-registering device* to indicate at every moment the actual delivery rate would be highly

desirable, and to this end the entire action of the mechanism should be positive so that the pump, when running at any particular speed shall inevitably move a corresponding volume of liquid. *The power and uniformity of action* should be such that a selected delivery rate may be sustained uniformly for 2 to 24 hours or more, with little attention in spite of variations of resistance such as may arise from changes of blood pressure or partial occlusions of tubing, cannulas, needles, or veins. *The accuracy* should be such that uncontrollable errors in the rate of delivery shall fall within the limits of error of methods available for detecting the physiological effects of such variations.

Parts coming into contact with the injection fluid should be quickly removable and replaceable without tools, for cleansing and sterilization. *The materials* should be selected with a consideration of their solubility in various injection media and their possible influence on the organism. Complex or delicate adjustments, valves, or other parts requiring undue care, or likely to become deranged or lost during ordinary use or cleaning, should be avoided.

Existing Types of Apparatus.

The various forms of apparatus described in the literature fall into three classes: (A) Devices in which pressure is imparted by gravity alone and the rate corrected by varying the size of the outlet. (B) Those in which pressure is imparted by a chamber of compressed air and the rate controlled as above. (C) Motor driven pumps.

It is unnecessary to consider in detail the disadvantageous features of apparatus of the first two types. The most notable use of a gravity device for prolonged intravenous injections was in the experiment of Henriques and Anderson,[1] who perfused a goat uninterruptedly for 20 days with a fair degree of accuracy. For a meter they used an ordinary dropping device inserted high up in the tube leading from the reservoir, a system which would not permit of a wide rate variation nor a change in the character of the liquid without recalibration. The principle of gravity flow, however refined and perfected, will necessarily involve

[1] Henriques, V., and Anderson, A. C., *Z. physiol. Chem.*, 1913, lxxxviii, 357.

cumbersome apparatus and considerable inconvenience, recommending itself only in special situations. Apparatus of Type B has been described by Kretschmer,[2] Rohde,[3] and others. Bulk, complexity, inconvenience, the undesirable solution of gases under pressure in the perfusion liquid, imperfect rate control, etc., are among their drawbacks. Motor driven pumps designed especially for organ perfusion work have been described by Brodie, Mandel, Friedmann, Straub, Kingsbury, and others. The problem of organ perfusion is not, however, identical with that of timed intravenous injections into the living animal. For perfusion purposes attention has been given especially to the injection of minute volumes of liquid in a pulsating stream, and especially by Kingsbury to materials and the form of the pulse wave. For the present problem, portability, adaptability to varying laboratory and bedside conditions, the convenient separation of parts for cleansing and sterilization, and above all positive, rapid, and exact regulation of rates through a wide range (*e.g.*, 10 to 2,000 cc. hourly), are features of prime importance. In the designs of perfusion pumps emphasis has not been laid on these points. On the other hand, the pulse form is of less significance for intravenous work.

Bock[4] was probably the first to use a motor driven pump for timed intravenous injections. His apparatus consisted of two glass syringes mounted side by side to form a duplex pump. The stems of the syringe plungers were screws and each passed through the center of a gear-wheel, as a bolt is screwed through a nut. When rotated, these wheels screwed the pistons in or out of the syringe barrels according to the directions of their rotation. The gear-wheels were turned in opposite directions by a single pinion engaging between them. Power was applied to the pinion by an electrical motor acting through a system of pulleys and belts. When either of the piston rods was screwed out to the end of a stroke it made an electrical contact, reversing the direction of the motor, and by the same device, a double three-way valve lying in front of the syringe barrels was operated by electromagnets. This complex apparatus delivered 0.2 to 10 cc. per

[2] Kretschmer, W., *Arch. exp. Path. u. Pharm.*, 1907, lvii, 428.
[3] Rohde, E., *Z. biol. Tech. u. Method.*, 1913, iii, 85.
[4] Bock, J., *Arch. exp. Path. u. Pharm.*, 1907, lvii, 177.

minute and was used in a study of renal function. The principle of reversing the motion of the pistons through the motor together with the belt transmission would necessarily involve irregularity and slippage. In 1913 Friedenthal and Willner demonstrated at the International Congress of Physiologists in Gröningen, a "*Praezisionsinjectorium*," concerning the details of which we have at present only the following program abstract: "The instrument serves the purpose of making possible the injection of specified solutions in the time desired and at the rate wished. It allows several experiments to be conducted simultaneously, or the injection of several solutions at different places into one animal. It permits further the preparation of unstable mixtures directly before injection. The instrument is also as appropriate for the most beautiful anatomic injections as it is for physiological and especially pharmacological and therapeutic injections." This is perhaps a later development of one of Friedmann's earlier perfusion pumps with multiple cylinders operated by a single machine, described in 1910,[5] an arrangement which would not be selected for the greatest exactness of rate control. In a report published in 1915[6] on the effects of prolonged and accurately timed intravenous injections of sugar are a cut and a brief description of a machine which has been used in this laboratory, consisting of a glass barrel and a metal piston driven by an electrical motor through worm and gear with a mechanically operated valve and a rheostat control. The model to be described presently represents merely a refinement of this machine. Raeder[7] has also mentioned using a motor driven Record syringe with a Bunsen valve to maintain even injections of salt solutions in a study of respiratory metabolism. The following cuts and descriptions are of an instrument built for us by William Gaertner and Company, of Chicago. Mr. Gaertner has embodied in it original solutions of some of the mechanical problems presented, such, for instance, as the link motion to meet the demand for a rapid means of adjusting the stroke of the pump, a feature which greatly increases the comfort of operation.

[5] Friedmann, E., *Biochem. Z.*, 1910, xxvii, 87.

[6] Woodyatt, R. T., Sansum, W. D., and Wilder, R. M., *J. Am. Med. Assn.*, 1915, lxv, 2067.

[7] Raeder, J. C., *Biochem. Z.*, 1915, lxix, 257.

New Apparatus.

The apparatus as a whole comprises two elements: (*a*) the pump and (*b*) the driving machine. Fig. 1 shows the pump unit taken apart for sterilization. Fig. 2 shows the same assembled and ready for insertion *en bloc* into the machine. Fig. 3 is a view of the machine from directly above showing the pump to one side of its bed on the driving mechanism in the position it would assume during detachment or replacement.

The Pump.—The pump (Figs. 1 and 2) consists of a metal piston with its rod, a ground glass cylinder, and a valve. On the extremity of the piston rod is seen the block by which the piston rod when in use is coupled to the carrier block of the driving machine (C in Fig. 3). The ground glass cylinder consists in this case of a 2 cc. Record syringe barrel with a special tip. The valve is seen to consist of a metal block perforated with a tapering hole and a tapering German silver core to fit. The principle of the valve, which is that of a three-way glass cock, is apparent from the cuts. In the center of the smaller end of the valve core is a screw projecting in the direction of its long axis. When the core is pushed into its place in the valve block, a spring washer is slipped over this screw and a circular milled nut screwed down to hold the block and core together with the proper tension. These details are visible in Fig. 1. On the lower end of the core, as seen in Fig. 2, is the slot to receive the valve lever by which the core is rocked to and fro, and the milled set nut to clamp the lever in place. It may be noted that the pump as a whole constitutes an interchangeable part which may vary in size and design and still be used on the same driving base. Thus, if it should be desired for special purposes to use the machine for timed injections of minute volumes of liquid, it would be possible to construct a special unit for this purpose. Since the stroke of the pump can be reduced literally to zero and the caliber of the barrel made as small as desired, the lower limit of the volume of liquid which can be pumped per minute depends wholly on the effectiveness of the valve.

The Driving Mechanism.—Power is provided by an electrical motor of $\frac{1}{40}$ horse power, and as it may be desired to use a single machine in different locations where the current available differs

in type, that shown in the cut (Fig. 3) is equipped with a universal motor which operates with any 110 to 120 volt current. For the best results, as with any motor, it is essential that the commutator be kept clean, and attention has been given to the details which make for the adequate protection and accessibility of this part. On the motor shaft in front of the motor casing is a heavy and carefully balanced leaden fly-wheel F, which adds greatly to the momentum and steadiness of the machine when running; and further forward the shaft is coupled through a flexible connecting piece with a worm running in oil on ball bearings. Only the protective housing of the worm is visible in the cut at W. Engaging with the worm is a large horizontal gear, the center of which is visible at G, while the remainder is likewise encased for protection. The gear-wheel shaft bearings are provided for in a heavy pillar which forms a part of the base casting of the machine. The gear-wheel revolutions are to those of the motor and worm as 1 to 200 (subject to change). The motion of the gear-wheel is transmitted to the piston of the pump through a system of links and levers designated on the cut as 1, 2, 3, 4, and 5, from which it is seen that one revolution of the gear-wheel corresponds to one complete oscillation of the piston. These links are so arranged that the length of the piston stroke may be set (if necessary, while the machine is in motion) at zero, 35 mm., or any intermediate value. This is effected at the lever 2 with the scale markings. The fixed pivot of this lever is beneath the end nearest the syringe under the letter S and on the lever is a sleeve (also S) which can be fixed in any position by a thumb screw. The link 3 is pivoted to the sleeve S and the length of the thrust it receives depends on the distance of the sleeve from the pivotal point of lever 2. The last link of the system 5 imparts its motion to a carrier block C which slides on a guide G. On this carrier block there is a screw with a milled head which serves to couple in place the block at the end of the pump piston when the apparatus is assembled. The valve of the pump is operated by an eccentric which lies beneath the gear-wheel. The eccentric is in fact part of the gear-wheel casting. Its motion is transmitted to the eccentric arm E (which is seen in the cut pivoted to the same pillar as the lever 4) and thence forward by a link to the valve lever proper. The left hand end of this link is visible but the valve lever does not show in the photograph.

It will be seen that in this machine the oscillations of the piston are directly proportional to the revolutions of the motor and cannot be otherwise. Also the valve is turned forcibly back and forth at the proper part of the stroke as an integral part of the motion of the whole machine. If the valve stops, the machine stops unless some part is broken or disconnected. Therefore, barring the factor of leakage in the valve and a wholly avoidable leakage about the piston or connections, the amount of liquid drawn into the pump and ejected during any interval is directly proportional to the number of strokes and this to the velocity of the motor. This would make it practical to provide the machine with a speed indicator for direct readings of the delivery rate at any moment, although this desirable factor has not been embodied in the present model. While the positive nature of the valve mechanism will naturally not insure against leakage due to imperfections in the valve itself, it has an advantage over delicate (ball, cone, or flap) valves in that the latter may be prevented from seating perfectly by gumming with thick liquids such as citrated blood, or from the lodgment of small particles between the valve surfaces. Leaks may thus develop during an experiment without affecting the speed of the machine and be detected only by timing the fall of the liquid in the supply burette. With a well made valve of the rotary type forcibly turned, this source of inconvenience is virtually eliminated and the machine becomes in reality a meter as well as a pump.

Performance.—The uniformity of the delivery rate with machines of the above design will depend practically on two factors: the perfection with which they are made, and the steadiness of the power current. An evenly balanced, smooth running motor, a true fly-wheel, well cut worm and gears, exact centerings and alignments, accurately fitted valves, proper oiling, etc., are of course essential. If these are satisfactory, the delivery rate is determined chiefly by the current and very little by variations of the pressure to be overcome in the tubing cannula, needle, or blood vessels. Two machines have now been subjected to heavy service in the wards and laboratory for a period of 12 months. One of them runs with great regularity and very quietly; the other is somewhat less quiet and requires more attention in order to secure even performances. They will aspirate liquids of vary-

ing viscosities such as water, citrated blood, or glycerol of 1.26 specific gravity, from burettes, flasks, beakers, etc., placed above or below them, and develop sufficient pressure to burst an ordinary rubber delivery tube if the latter is clamped while the machine is running, and this without materially slowing the rate of the machine. The stream emerging from an open delivery tube intermits twenty to forty times per minute according to the number of strokes. When the delivery tube terminates in a needle or cannula, the elasticity of the wall tends to equalize the pressure and an undulating stream results. The stream can be made continuous and uniform by the use of a section of thin-walled tubing with a narrow outlet or other equalizing device. For intravenous work it has proved satisfactory. A single machine has been run continuously for 19 hours by Sansum in actual animal experimentation, and they might be run for days. It has not been found difficult to make the quarter hour and hour discharge fall within 1 per cent of the desired values. With a favorable current and perfectly constructed machine, no attention whatsoever may be required for hours at a time to secure a uniform rate of delivery; but if the current fluctuates, or if the machine is imperfectly made or cared for, more attention will be necessary. By the rheostat alone the rate can be varied to a nicety between twenty and forty strokes to the minute with an ample working margin above and below. When the barrel of the pump has a bore of 8 to 9 mm., or about that of an ordinary 2 cc. all glass hypodermic syringe, the delivery rate may be varied by changing both stroke and speed from 1 cc. to 80 cc. per minute (60 to 4,800 cc. per hour). If higher rates of injection or aspiration were called for, which seems improbable, a larger barrel could be used, while the use of a barrel corresponding in size to a 0.5 cc. syringe permits of injection at rates as low as 0.2 cc. per minute (12 cc. hourly). The machine could be adapted readily for injections of smaller volumes per minute, but this has not been done with the present instruments. By the use of two (or more) machines with the discharging needle of one (or more) thrust into the delivery tube of another, or by the use of two or more machines injecting simultaneously into different sites in the same animal, a great variety of experimental combinations is made possible. We have, for example, injected a standard glucose solution at a

uniform rate with one machine, while with a second discharging into the delivery tube of the first, pure water, salt solutions, etc., have been superadded at specified rates. The power of aspirating fluids as well as injecting them, and to aspirate if desired at known rates, holds several possibilities.

There has been practically no inconvenience arising from occlusions of the needles, cannulas, and veins, except when the solution itself has been injurious to the vessel lining, even when injections have been made for long periods into small veins, such as the ear veins of a rabbit.

The present apparatus is doubtless subject to variation and further improvement. For special purposes, and where portability is not a factor, other mechanical solutions of the problem might be preferred, but the model described combines several qualifications of an ideal instrument and it has proved a valuable addition to the equipment of the ward and laboratory.[8]

EXPLANATION OF PLATES.

PLATE 2.

FIG. 1. The pump unit taken apart, as for sterilization.

FIG. 2. The parts shown in Fig. 1 assembled and ready for insertion into the driving machine.

PLATE 3.

FIG. 3. The machine from directly above, showing the pump to one side of its bed on the driving mechanism in the position it would assume during detachment or replacement.

F. Fly-Wheel.
W. Worm housing.
G. Exposed center of gear-wheel.
1, 2, 3, 4, 5. Links and levers which transmit power from gear-wheel to pump.
S. Movable sleeve on lever 2 for regulation of the length of the stroke.
C. Carrier block with post for fixation of the outer end of the pump piston.
G^1. Guide on which the carrier block slides.
E. Eccentric arm for valve operation.
P. Insertion point for electrical plug.

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[8] Previous references to this pump have been made by Woodyatt, Harvey Lecture, 1916, and Wilder, R. M., and Sansum, W. D., *Arch. Int. Med.*, 1917, xix, 311. Later reports deal further with application of these principles and apparatus.

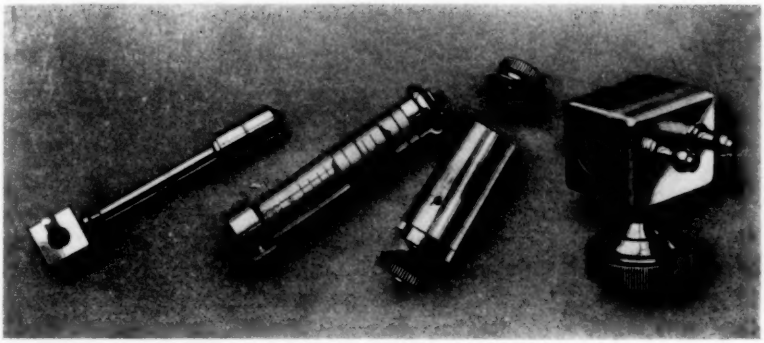

FIG. 1.

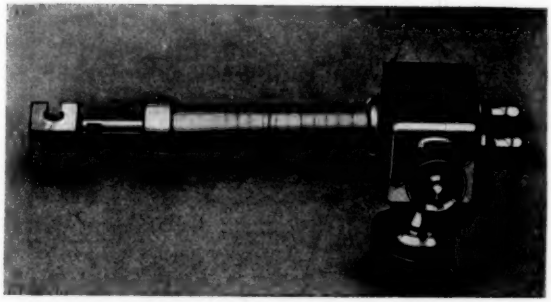

FIG. 2.

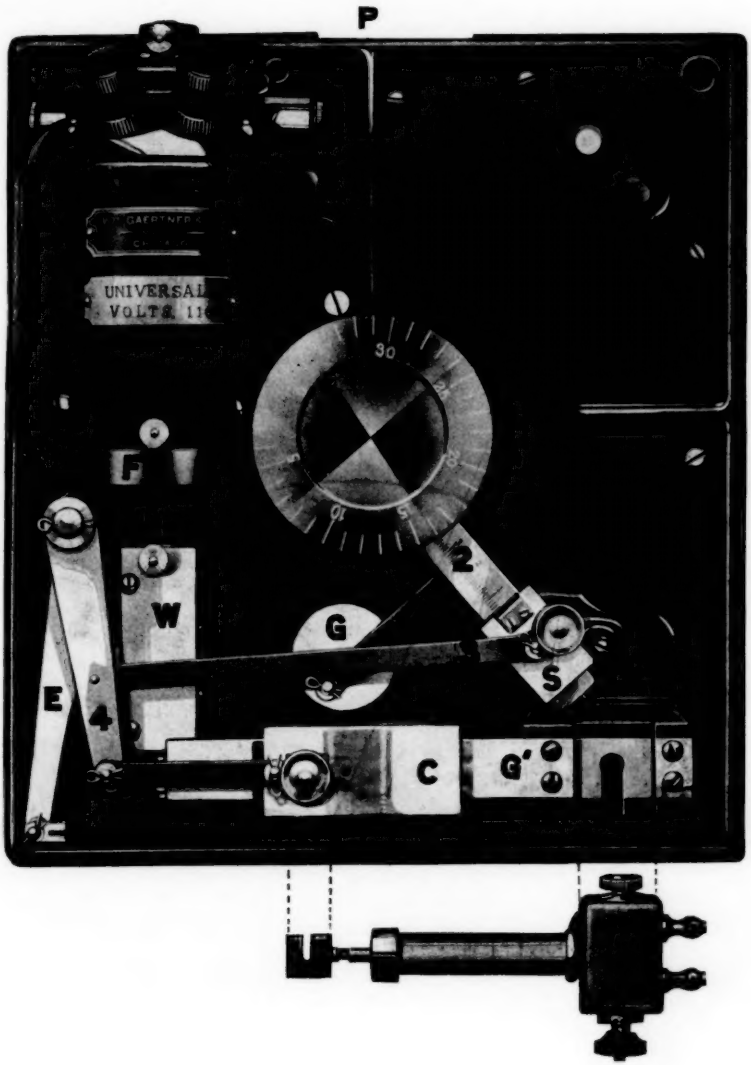

FIG. 3.

(Woodyatt: Method of Injection.)

THE EFFECT OF BILE AND BILE SALTS ON THE REACTION BETWEEN OLEIC ACID AND SODIUM BICARBONATE.*

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More than half of the fat ingested by an animal is absorbed from the small intestine as an aqueous solution of its fatty acids, or of its soaps, or of both, and this solution, of fatty acids in particular, is effected by the bile.

It has been shown by Strecker (1), Marcet (2), Latschinoff (3), and Kühne (4), and confirmed by Moore and Rockwood (5) that fatty acids are soluble in bile. It was shown by Moore and Parker (6) that oleic, palmitic, and stearic acids, and their sodium, calcium, and magnesium salts were practically insoluble in 5 per cent bile salt solutions unless these solutions contained 1 per cent of lecithin.

Pflüger (7), assuming that the alkali of the intestine, available for the neutralization of fatty acids, is sodium carbonate, attempted to show quantitatively the effect of bile on this neutralization. His method was to mix bile, sodium carbonate solution, and oleic acid or other fatty acids, the latter being present in amounts equivalent to the alkali, and after allowing the mixture to stand at 37° for several hours to extract the remaining free fatty acid by ether, and to dry and weigh it. In this way he found in one experiment that without bile the neutralization was 19 per cent of the oleic acid used, but in the presence of bile it was 10.57 per cent only. The presence of bile had decreased the ability of the two substances to react.

These low neutralization values which Pflüger found are easily explained by the observation of Chevreul that soaps hydrolyze in water solutions yielding free alkali and free fatty acid. This fatty acid from the hydrolyzed, preformed soap may be extracted by a fat solvent, and, therefore, in determining the amount of a fatty acid in a mixture containing

* The major part of this work was included in a thesis for the degree of Doctor of Philosophy, accepted by the Division of Medical Sciences of Harvard University.

soap it is necessary to prevent the hydrolysis of the soap. In a later investigation Pflüger (8) found that neutral oleate yielded 24.5 per cent of its combined fatty acid as free fatty acid when thoroughly extracted by ether, but did not apply this large correction to his earlier work or admit his error. Pflüger's experiments have been repeated, but with different results, as will be shown later.

Pflüger's assumption that the alkali of the small intestine available for the neutralization of fatty acids is sodium carbonate must be regarded as entirely without foundation in fact, as the following evidence shows.

Auerbach and Pick (9) measured the hydrogen ion concentration of the intestinal juice of dogs and found it to be 1×10^{-8} normal, and taking into consideration the disturbing effect of the small amount of carbon dioxide present, assumed that this was only a minimal value and that the true value is higher, 2×10^{-8} normal, which is little less than that of blood. These results are confirmed indirectly by the optimum hydrogen ion concentrations for trypsin, 2×10^{-8} normal, for pancreatic lipase, 1 to 2×10^{-8} normal, and for erepsin, 2×10^{-8} normal.

McClendon (10) found that the hydrogen ion concentration of the duodenal contents of normal men is 1 to 5×10^{-8} normal. These values indicate that the reaction of the small intestine is very faintly alkaline, corresponding to a dilute solution of sodium bicarbonate, perhaps containing a little free carbon dioxide, but not under any circumstances to a solution of sodium carbonate, no matter how dilute (11).

Although Pflüger's work in this field is given a prominent place in the text-books of physiological chemistry, a careful study of his papers shows that he started on a wrong assumption and that much of his experimental work was incorrect.

Since the alkali of the small intestine can be regarded as sodium bicarbonate, the extent of the soap formation during the digestion of fats will depend upon the ability of this alkali to neutralize fatty acids. It is the purpose of this paper to show by experiments *in vitro* that the presence of bile or bile salt solutions greatly accelerates this neutralization, with the formation of more soap (in some cases several times as much) than is formed in the same time and under comparable conditions when these substances are not present.

EXPERIMENTAL.

The problem was attacked in two ways, first by determining the amount of unused fatty acid (oleic acid was taken as a representative fatty acid) after it had been treated with sodium bicarbonate, and second by measuring the carbon dioxide given off in the reaction.

Materials.

Oleic acid was made from a high grade olive oil by saponification with alcoholic soda, setting free the fatty acids by the addition of dilute sulfuric acid, and subsequently separating the oleic acid by the lead soap procedure. The acid so obtained had an iodine number 96.9, and an acid number 196, as compared with 90.07 and 198, the iodine and acid numbers of the pure substance. It was nearly as colorless as water and was used in all the experiments unless otherwise specified.

Sodium oleate was made by exactly neutralizing oleic acid in alcohol solution with alcoholic soda, with phenolphthalein as indicator, and removing the alcohol by evaporation. The dry soap in absolute alcohol solution reacted neutral to phenolphthalein, 1 gm. of it requiring only one drop of 0.1 N sodium ethylate to make it distinctly alkaline.

First Method. Prevention of the Hydrolysis of Soap.

Solutions of sodium oleate in water were made up to the following concentrations: 0.1, 0.2, 0.3, 0.5, and 1.0 per cent. 100 cc. of these solutions were saturated by shaking with an excess of solid sodium chloride (40 gm.). The mixture was then extracted with 100 cc. of neutral chloroform in four portions. The filtered extract with the rinsings from the filter paper was titrated against 0.1 N sodium ethylate and the degree of hydrolysis determined. The results are shown in Table I.

TABLE I.

Free oleic acid in terms of soap.	Hydrolysis.	Soap in 100 cc. of solution.
gm.	*per cent*	*gm.*
0.0162	16.2	0.10
0.0192	9.6	0.20
0.0236	7.8	0.30
0.0280	5.6	0.50
0.0310	3.1	1.00

It is seen from Table I that, if care is taken to have the concentration of the soap as great as 1.0 per cent, the hydrolysis, as determined by this method, is a matter of only 3.1 per cent as

compared with that found by Pflüger (8) of 24.5 per cent for the oleate and 21.4 per cent for the palmitate when hydrolysis was not prevented.

The general plan of the experiments was as follows: 5 cc. of oleic acid (4.353 gm.) were pipetted into a 200 cc. Erlenmeyer flask, and known amounts of sodium carbonate or bicarbonate and 25 cc. of fresh, filtered ox bile were added. In the cases in which bile was not used an equivalent volume of water was substituted. Additional amounts of water were then added to make the contents up to 100 cc., exclusive of the oleic acid. The flask was shaken for a minute and put in the warm room at 37.5° for 15 to 20 hours. At the end of that time the mixture was saturated with solid sodium chloride and extracted with neutral chloroform as previously described. The filtered extract was made up to 250 cc., 25 cc. aliquots were titrated against 0.1 N sodium ethylate, and the amount of neutralization was determined. The results are shown in Table II.

TABLE II.*

No.	Temperature.	Water.	Bile.	$NaHCO_3$	Na_2CO_3	Oleic acid recovered.	Combined.	Free.
	°C.	cc.	cc.	gm.	gm.	gm.	per cent	per cent
2	37.5	100		1.298		3.238	25.5	74.5
3	37.5	200			0.8188	2.658	38.9	61.1
4a	37.5	75	25			4.210	3.3	96.7
4b	37.5	75	25			4.263	2.1	97.9
6	37.5	75	25			4.010	7.8	92.2
7	37.5	75	25	0.50		2.787	35.9	64.1
8	37.5	100		0.50		3.832	11.9	88.1

* 5 cc. of oleic acid (4.353 gm.) were used in each experiment.

As shown in Table II, amounts of sodium bicarbonate and carbonate equivalent to the oleic acid were used in Experiments 2 and 3. A consideration of these experiments shows that the neutralization of oleic acid by sodium bicarbonate is 25.5 per cent only. With sodium carbonate there is a greater soap formation, 38.9 per cent, but in neither case is the reaction complete. In an experiment similar to Experiment 3, Pflüger found that the neutralization with sodium carbonate was only 19 per cent, but it has already been noted that this was due to the extraction of

an excess of oleic acid from the preformed but hydrolyzed sodium oleate.

A comparison of Experiments 7 and 8 shows that the presence of bile makes the reaction with sodium bicarbonate more complete than is otherwise the case. That this cannot be accounted for by the alkali of the bile itself is shown by the low neutralization values obtained in Experiments 4 a, 4 b, and 6.

Experiments 7 and 8 were repeated using 20 cc. of a 10 per cent barium chloride solution. The use of this reagent entirely prevented the hydrolysis of the preformed soap, as shown by the fact that oleic acid could be quantitatively determined in the presence of sodium oleate. This was learned from several experiments. The chloroform extract was boiled to remove traces of carbon dioxide which are easily extracted from mixtures containing it and possess titratable values. The results are shown in Table III.

TABLE III.

No.	Bile.	Combined oleic acid.	Free oleic acid.
	cc.	*per cent*	*per cent*
7a	25	53.7	46.3
8a		27.6	72.4

Table III shows that the increase in the amount of oleic acid neutralized by sodium bicarbonate in the presence of bile over that without bile was 26.1 per cent. In Experiment 7 there was a similar increase of 24.0 per cent. The effect of bile is marked and is exactly the opposite from that found by Pflüger in a similar experiment with sodium carbonate, bile, and oleic acid.

Attempts to use this method of analysis with bile salts in place of bile failed because of the troublesome emulsions formed. The second method, that of determining the carbon dioxide production as an index of soap formation, was therefore resorted to.

Second Method.

Air was drawn through a tower (A) containing solid potassium hydroxide into a cylinder (B), 23 cm. high by 5 cm. internal diameter, having a slightly constricted opening fitted with a three

hole rubber stopper. The entrance tube from (A) reached to the bottom of the cylinder and had a perforated bulb at the lower end to decrease the size of the entering air bubbles. Nearly at the top of this tube, just under the stopper, was placed a rubber washer to prevent the froth from entering the exit tube. The stem of a small dropping funnel was inserted through the second hole. The third hole of the stopper contained the exit tube which was connected to a smaller cylinder (C) one-quarter full of concentrated sulfuric acid; and this in turn was attached to a calcium chloride U-tube (D). The calcium chloride in this tube had been saturated with carbon dioxide and the excess removed by washing with carbon dioxide-free air. The U-tube (D) was directly connected with a potash bulb of the Wetzlar type guarded from back suction of air and water by a large U-tube (F) containing solid potassium hydroxide and calcium chloride in alternate layers.

Air was drawn through the apparatus by connecting the U-tube (F) with a large aspirating bottle from which the water flowed by gravity into a large graduated cylinder. In order that the experiments might be made comparative, the rate of flow of the carbon dioxide-free air which ventilated the apparatus was made as uniform as possible in the several experiments.

The sodium bicarbonate solution was approximately 0.5 N and was standardized in the apparatus by adding an excess of 10 per cent sulfuric acid slowly from the dropping funnel in the cylinder (B) and drawing air through the apparatus until the weight of the potash bulb remained constant. 5 cc. of the bicarbonate solution were used in each experiment. 5 cc. of Merck's oleic acid, an amount in excess of the bicarbonate, were used.

Bile salt was prepared from ox bile according to the method of Tengström (12), and was that fraction obtained from the ferric chloride precipitate by decomposing it with sodium carbonate. The bile salt was completely separated from the contaminating sodium chloride and carbonate by repeated extractions with absolute alcohol. Analysis showed that the salt contained a small amount of sulfur and was therefore glycocholate slightly contaminated with taurocholate. A 2 per cent solution was used. This reacted neutral to litmus and phenolphthalein, but slightly alkaline to alizarin red. 10 cc. of the solution could be made

distinctly acid to this indicator by the addition of 0.05 cc. of 0.1 N hydrochloric acid.[1]

Certain bile salts were found to be soluble in oleic acid, as is also bile itself under certain conditions, and these solutions crystallize out on standing. In one experiment a solution of bile salt in oleic acid was used.

Shark bile salt, the fraction not precipitable by ferric chloride, and therefore corresponding in properties to the taurocholate of ox bile, was used in one experiment.

In two experiments lecithin and sodium oleate were substituted for bile salt.

Method of Procedure.

5 cc. of oleic acid were pipetted into the dry reaction cylinder (B) with 15 cc. of bile or bile salt solution and carbon dioxide-free air drawn through the apparatus with the potash bulb in place until all the carbon dioxide from the carbonates (if bile was used) had been removed. This preliminary procedure was found unnecessary with bile salt solutions.

The cylinder (B) was opened, 5 cc. of bicarbonate solution were quickly introduced by a pipette, and the cylinder was closed. A known volume of air was then drawn through the apparatus and the time recorded. The stop-cocks on the tube (D) were then closed, preventing the escape of carbon dioxide, the potash bulb was weighed as quickly as possible, and immediately put back in place. This operation was repeated until the weight of the potash bulb remained constant after two or three periods. All experiments were done at room temperature.

[1] On standing a short time a 2 per cent solution of sodium glycocholate becomes turbid and a flocculent precipitate falls out. If a fresh solution is immediately diluted with water to 0.2 per cent, it becomes milky and in a short time a small quantity of crystals forms. This is thought to be due to a hydrolysis of the salt with the formation of free bile acid, since the crystals were difficultly soluble in hot water and exhibited other characteristics of bile acid. A trace of alkali prevents this precipitation or crystallization.

PROTOCOL

Total time elapsed.	Total volume of aspirated air.	Total CO_2 evolved.
1.* 10 cc. water. 5 cc. $NaHCO_3$. 10 cc. 10 per cent H_2SO_4.		
min	*cc*	*mg*
15	1,000	67
36	1,700	98
190	4,700	108
290	5,700	109
320	6,600	109
2.* 15 cc. water. 5 cc. $NaHCO_3$. 5 cc. oleic acid.		
30	2,300	6
80	4,600	11
202	6,200	20
387	10,550	35
697	15,450	48
3. 15 cc. undiluted ox bile. 5 cc. oleic acid. Partial emulsion.		
30	2,000	12
85	3,500	12
5 cc. $NaHCO_3$ solution added.		
20	1,150	80**
50	2,350	103**
95	3,400	106**
130	5,025	108**
210	8,225	108**
4. 15 cc. 10 per cent bile (containing about 1 per cent bile salt). 5 cc. oleic acid. 3,000 cc. of air used to remove the trace of CO_2 from the bile, and 5 cc. $NaHCO_3$ solution added.		
17	1,250	56
42	1,850	75
79	3,250	95
149	4,550	103
203	5,550	103

* This experiment was repeated and the results were confirmed.

** Exclusive of the 12 mg. of CO_2 from the bile.

PROTOCOL—*Continued.*

Total time elapsed.	Total volume of aspirated air.	Total CO_2 evolved.
5. 15 cc. 5 per cent bile (containing about 0.5 per cent bile salt). 5 cc. oleic acid. 5 cc. $NaHCO_3$ solution. Partial emulsion.		
min.	*cc*	*mg.*
17	950	22
43	1,700	52
84	2,800	76
190	4,800	97
247	6,400	102
317	8,700	104
372	9,600	104
6. 15 cc. 1 per cent bile (containing about 0.1 per cent bile salt). 5 cc. oleic acid. 5 cc. $NaHCO_3$ solution. Very slight emulsion.		
15	850	12
44	1,750	33
79	3,220	62
189	5,200	84
229	6,500	92
264	7,450	95
394	9,950	100
7. 15 cc. Na glycocholate, 2 per cent solution. 5 cc. oleic acid. 5 cc. $NaHCO_3$ solution.		
15	1,000	42
34	1,750	72
125	2,700	102
172	6,450	106
212	8,050	106
250	9,550	106
8. 15 cc. 1.3 per cent shark bile salt. 5 cc. oleic acid. 5 cc. $NaHCO_3$ solution. Blank determination on bile salt and oleic acid gave no CO_2.		
15	800	33
35	1,850	76
100	3,350	101
150	4,975	106
185	6,475	106

PROTOCOL—*Concluded.*

Total time elapsed.	Total volume of aspirated air.	Total CO_2 evolved.
9. 15 cc. water. 5 cc. oleic acid. 0.2 gm. lecithin (Merck's). 5 cc. $NaHCO_3$ solution.		
min.	*cc.*	*mg.*
15	1,050	9
40	1,925	21
191	3,925	49
10. 15 cc. water. 5 cc. oleic acid containing dissolved bile salt. 5 cc. $NaHCO_3$ solution. Partial emulsion.		
15	1,000	52
35	1,850	90
100	3,050	95
168	4,650	102
198	5,250	103
228	6,100	104
268	7,100	104
11. 15 cc. 1 per cent Na oleate solution. 5 cc. oleic acid. 5 cc. $NaHCO_3$ solution. Partial emulsion.		
19	1,400	9
45	2,400	16
115	4,200	29
180	5,500	37

Explanation of Charts.

No. of experiment.				
1	10 cc. 10 per cent H_2SO_4,	5 cc. $NaHCO_3$ solution,	15 cc. water.
2	5 " oleic acid,	5 " " "	15 " "
3	5 " " "	5 " " "	15 " ox bile.
4	5 " " "	5 " " "	15 " 10 per cent ox bile.
5	5 " " "	5 " " "	15 cc. 5 per cent ox bile.
6	5 " " "	5 " " "	15 cc. 1 per cent ox bile.
7	5 " " "	5 " " "	15 cc. 2 per cent bile salt.
8	5 " " "	5 " " "	15 cc. shark bile salt.
9	5 " " "	5 " " "	15 cc. 1.3 per cent lecithin.
10	5 " " "	5 " " "	15 cc. water + bile salt in oleic acid.
11	5 " " "	5 " " "	15 cc. 1 per cent Na oleate.

Charts A, B, and C show the results of these experiments graphically. It will be noted that the curve of the velocity of the reaction between sulfuric acid and sodium bicarbonate under the experimental conditions is similar to that of oleic acid on sodium bicarbonate in the presence of undiluted bile, as shown by Curves 1 and 3.

A comparison of Curves 2, 3, and 7 shows how marked is the effect of bile or bile salt. A 2 per cent solution of sodium glycocholate is not so effective as bile which contains approximately 10 per cent of bile salts, as is to be expected. Curves 4 and 7 show that 10 per cent bile, in which the concentration of bile salt is approximately 1 per cent has the same effect as a 2 per cent solution of the bile salt itself. This added effect of bile is probably due to the presence of pseudo mucin, since Moore and Rockwood (5) found that the removal of this substance from bile rendered the latter practically incapable of dissolving fatty acids. Lecithin and sodium oleate increase the velocity of this reaction,

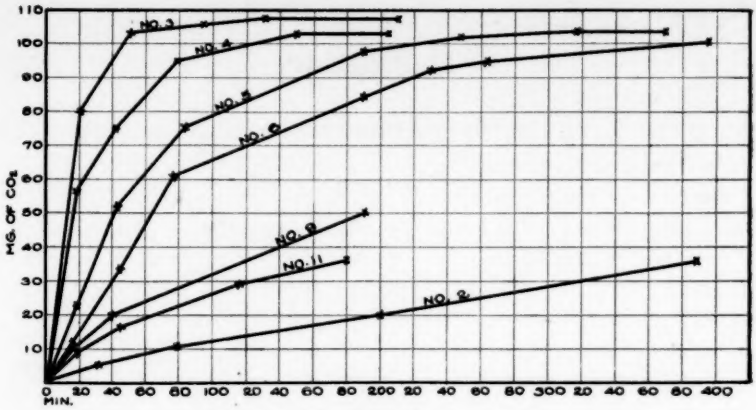

CHART A.

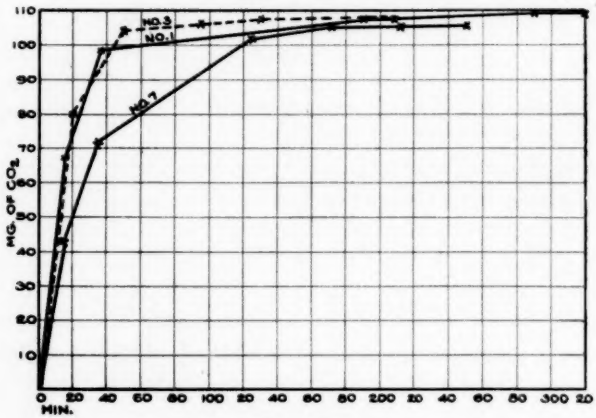

CHART B.

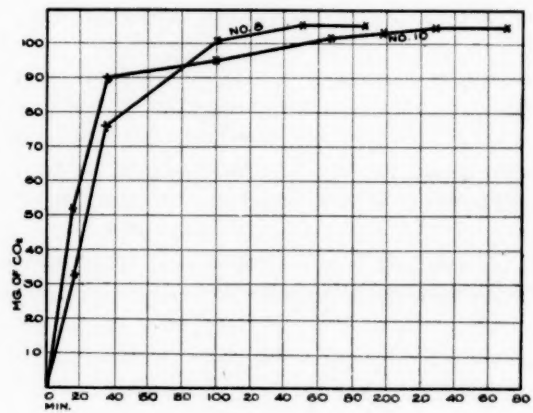

CHART C.

but are not so effective as 1 per cent bile, having a concentration in bile salt of approximately 0.10 per cent, shown by Curves 6, 9, and 11. Moore and Parker (6) found that bile salt itself had almost no solvent effect on fatty acids, but that if it contained 1 per cent of lecithin its solvent effect was comparable to that of bile. Shark bile salt has the same effect as ox bile salt, shown by Curve 8. Bile salt dissolved in oleic acid acts similarly to the same substance dissolved in water, shown by Curve 10.

The effect of bile is strikingly shown by a comparison of Experiments 2 and 3. In the former the soap formation corresponds to 48 mg. of carbon dioxide in 11 hours. In the latter, in the presence of bile, the soap formation corresponds to 80 mg. of carbon dioxide in 20 minutes.

CONCLUSION.

While it is dangerous to apply conclusions from simple experiments of this kind to the actual conditions in the small intestine during the digestion and absorption of fat, it seems reasonable to believe that the presence of bile makes possible a much greater soap formation from the fatty acids liberated during digestion than could otherwise be the case with an alkali as weak as sodium bicarbonate. A consideration of the physical properties of bile and bile salt solutions would lead one to expect the results obtained, but it seems of value to have demonstrated this experimentally. Attention is called to the fact that all of Pflüger's work in this field, published in several papers and cited in textbooks of physiological chemistry, is based on the erroneous assumption that the alkali of the small intestine, available for the neutralization of fatty acids, is sodium carbonate; and that much of his experimental work, based on this wrong assumption is, in his earlier papers at least, incorrect, because of an inadequate analytical procedure.

I am grateful to Professor Otto Folin, who suggested this problem, for many helpful suggestions and a constant interest in the work.

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VARIATIONS IN THE COMPOSITION OF HUMAN MILK DURING THE FIRST ELEVEN DAYS AFTER PARTURITION.

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By a study of the variations in the composition of human milk during the first few days of lactation it is possible to assist in an understanding of the metabolic changes taking place when the organism takes upon itself the function of food production as distinct from the elaboration of another individual. That this change in function must be accompanied by deep-seated metabolic changes is obvious. Hence if it is possible to trace regular variations in the most evident milk constituents, these variations may be traced to related processes in the mother.

Although innumerable analyses have been made to determine the per cent of protein, fat, and lactose in milk, there is a definite lack of information as to their interrelationship and variation in the same individual on several successive days.

The literature up to 1874 is thoroughly reviewed by Gorup-Besanez (1). The nearest approach to work of this nature is the report of Clemm (2) obtained from the study of one subject. Tidy (3) gives the average of analyses of composite samples taken during the first week after parturition. Camerer and Söldner (4, 5, 6), in a series of three papers, give the results of their analyses of fifty-seven samples of the composite milk from many women, and tabulate them with respect to the time after parturition. Meigs and Marsh (7), following the example of Tidy, report on the analyses of five composite samples taken between the 5th and 9th days after parturition. The information presented in these papers is of great value and serves as a standard by which other results of a similar general nature can be compared. They are, however, lacking in detail of advantage to the present problem.

Vernois and Becquerel (8), l'Heretiér (9), Kühn (10), Lehmann (11), Hinchcliff (12), Farrington (13), Penny (14), Eckles and Shaw (15), Eckles and Palmer (16), and others have demonstrated that in general the pro-

duction of cow's milk is influenced by various factors, such as breed, constitution, age, feed, stage of lactation, interval between milking, individuality, disease, etc. Notwithstanding this multiplicity of interfering factors the basic principles upon which milk production is accomplished are stable, and it is not illogical to suppose that a certain uniform variability in composition can occur during the progress of adjustment. It is the purpose of this report merely to point out these regularities in variation since sufficient data are not yet at hand to permit definite conclusions as to their cause.

The subjects of this investigation were healthy patients in the Los Angeles County Hospital. The diet was the same for all throughout the period of study. The milk production of eight women was studied.

Case.	Date. 1916	Case.	Date. 1916
1	Aug. 30–Sept. 7	5	Nov. 3–11
2	Oct. 6–14	6	" 3–11
3	" 17–25	7	" 5–13
4	" 17–25	8	" 5–13

The milk was collected by means of the breast pump at the same hour on the 3rd, 5th, 7th, 9th, and 11th days after parturition. Inasmuch as the patients are dismissed from the hospital the 12th day after delivery, milk collected thereafter would have been subjected to the influence of diet variation.

The total nitrogen, fat (lipoids), and lactose were determined in the fresh milk as sent to this laboratory immediately after collection.

The total nitrogen was estimated by the microchemical modification of Kjeldahl's digestion, devised by Folin and Farmer (17). The fat was determined by Meigs' (7) method. The lactose was determined in the protein-free filtrate by the use of Fehling's solution, with occasional polariscopic corroboration. Acetic acid, lead acetate, and heat were the protein precipitants used. All results are calculated to the basis of gm. of constituent in 100 gm. of milk.

Total Nitrogen.

In looking over the figures published by various investigators it seems as if the milk produced by the women of this country during the first 11 days of lactation is somewhat lower in total nitrogen content than is that of those abroad.

TABLE I.

A Comparison of the Averages from the Total Nitrogen Determinations in Milk of the 5th to the 11th Days after Parturition.

Total nitrogen.	Source.	Author.
per cent		
0.278	Average of the analyses of 13 composite samples.	Söldner (4). Camerer and Söldner. (5, 6).
0.244	Average of the analyses of 5 composite samples.	Meigs and Marsh (7).
0.255	Average of the analyses of 31 individual samples.	Hammett.

Further variations in nitrogen content are considered together with the variations in protein production.

The figures of Söldner and Camerer are taken from their third paper wherein they collect into one table the results of their whole series of analyses.

Protein.

Much work has been carried on in an attempt to differentiate between the various forms of nitrogen-containing substances in milk in order that a true picture of the protein output can be calculated. A discussion of this phase is beyond the scope of the present paper. While Camerer and Söldner (4, 5, 6) use the factor 6.25, and Eckles and Shaw (15) 6.38, Hawk (18) advocates the use of 6.37 as the figure by which to multiply the total nitrogen in milk in order to obtain the protein production. This last factor has been used in our calculations.

In Table II will be found the per cent of protein in the milk of eight women for the 3rd, 5th, 7th, 9th, and 11th days after parturition.

Group Variations for Each Day.—An inspection of the table will show that the limits within which the individual protein production may occur suffer a marked narrowing between the 3rd and the 5th days of lactation, the subsequent variation limits decreasing more gradually until on the 11th day the maximum variation from the mean is ± 18 per cent.

TABLE II.

The Per Cent of Protein and Total Nitrogen in Human Milk on the 3rd, 5th, 7th, 9th, and 11th Days after Parturition.

Case.	1		2		3		4		5	
Day.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.
3	1.99	0.312	5.79	0.908	4.81	0.755	2.06	0.322	5.09	0.800
5	1.62	0.255	1.97	0.310	1.90	0.298	1.58	0.248	2.32	0.364
7	1.49	0.235	1.83	0.287	1.65	0.260	1.60	0.252	1.92	0.301
9	1.91	0.307	1.77	0.277	1.70	0.267	1.55	0.244	1.82	0.286
11	1.64	0.258			1.59	0.250	1.41	0.221	1.38	0.216
Average	1.73	0.271	2.84	0.453	2.33	0.365	1.64	0.257	2.50	0.392

Case.	6		7		8		Average.		High.		Low.	
Day.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.
3	3.64	0.570	1.82	0.286	2.96	0.465	3.52	0.552	5.79	0.908	1.82	0.286
5	1.66	0.261	1.57	0.247	1.29	0.203	1.74	0.274	2.32	0.364	1.29	0.203
7	1.38	0.216	1.38	0.216	1.64	0.258	1.61	0.254	1.91	0.301	1.38	0.216
9	1.40	0.220	1.47	0.231	1.78	0.279	1.69	0.264	1.93	0.307	1.40	0.220
11	1.35	0.212	1.18	0.184	1.70	0.267	1.46	0.229	1.70	0.267	1.18	0.184
Average	1.88	0.295	1.49	0.233	1.87	0.293	2.00	0.314	2.73	0.428	1.41	0.222

Group Variations from Day to Day.—Coincident with the narrowing of the limits within which the protein production occurs there is a decline in the actual per cent of protein content of the milk, and it is during the period of most marked decrease in divergency that the most evident decrease in protein content takes place.

Individual Variations from Day to Day.—These show the same tendency towards a decrease with the progress of lactation.

Individual Variations from the Group Average for the Single Days.—From a qualitative point of view the individual variation from the group average for each day tends to be uniformly above or below that figure. This leads to the conclusion that the protein production plane is fixed for the individual, is independent of the plane of nutrition, and is dependent on the individuality, thus fundamentally correlating the findings of Eckles and Palmer (16) in their studies on the milk production of the cow.

Fat.

All reports agree that the most variable milk constituent is the fat. Hence it is not surprising that a comparison of the averages of various composite samples shows no consistent variations.

TABLE III.

A Comparison of the Averages from the Fat Determinations in Milk of the 5th to the 11th Days after Parturition.

Fat.	Author.
per cent	
3.18	Söldner, Camerer and Söldner (4, 5, 6).
2.45	Meigs and Marsh (7).
3.25	Hammett.

In Table IV there is a tabulation of the results of the fat determinations on the milk of the eight subjects studied.

TABLE IV.

The Per Cent of Fat in Human Milk on the 3rd, 5th, 7th, 9th, and 11th Days after Parturition.

Day.	1	2	3	4	5	6	7	8	Average.	High.	Low.
3	2.41	2.84	4.14	1.92	12.70	3.99	3.51	3.20	4.34	12.70	1.92
5	2.61	2.80	2.79	2.12	3.69	4.26	1.89	2.86	2.88	4.26	1.89
7	3.97	5.79	1.81	2.98	2.50	4.21	3.83	5.28	3.80	5.79	1.81
9	4.67	4.15	2.28	2.62	2.85	3.88	4.08	5.11	3.70	5.11	2.28
11	3.37		2.44	1.92	3.08	3.93	5.02	3.89	3.38	5.02	1.92
Average..	3.41	3.92	2.69	2.31	4.98	4.06	3.67	4.07	3.62	6.58	2.00

Group Variations for Each Day.—If we except the unusually high fat per cent found in the milk from Case 5 on the 3rd day there are no regularly occurring changes in the limits within which the fat per cent of human milk may be found during the early days of lactation. On the 11th day the maximum variation from the mean is ±44 per cent, a much greater divergency than that present in the protein production.

Group Variations from Day to Day.—From a quantitative standpoint the averages published by Camerer and Söldner (6)

and the results herein reported show an increase in the fat production after the 3rd day.

Individual Variations from Day to Day.—These show no tendency towards a diminution.

Individual Variations from the Group Average for the Single Days.—If we consider the direction of variation from the average, it is demonstrated that here as well as with the protein production there exists an individual tendency to uniformity of production plane. Inasmuch as the fat production is individually so extremely variable it is not surprising that the level of the production plane fails to maintain the same degree of consistency as is shown by the protein. Nevertheless the tendency is present and is of sufficient degree to afford still further evidence that the principle stated for protein production holds good in a like manner for the fat production of the lactating woman.

Lactose.

The average lactose content of human milk from the 5th to the 11th days after parturition according to various published reports is found in Table V. As far as these figures go they show that the American investigators find a higher sugar production during the early days of lactation than do those abroad. If the differences are valid they are significant of a higher lactose production plane in the subjects of the former workers.

TABLE V.

A Comparison of the Averages from the Lactose Determinations in Milk of the 5th to the 11th Days after Parturition.

Lactose.	Author.
per cent	
3.86	Gorup-Besanez (1), Clemm.
5.14	Gorup-Besanez (1), Tidy.
5.99	Camerer and Söldner (6).
6.53	Meigs and Marsh (7).
6.17	Hammett.

Table VI gives the results of the analyses for lactose of the samples of milk from the eight women studied.

TABLE VI.

The Per Cent of Lactose in Human Milk on the 3rd, 5th, 7th, 9th, and 11th Days after Parturition.

Day.	1		3	4	5	6	7	8	Average.	High.	Low.
3	6.49	6.20	4.63	6.51	2.31	5.72	6.11	5.44	5.43	6.49	2.31
5	6.44	4.15	6.56	6.81	4.96	6.52	6.86	6.33	6.08	6.86	4.15
7	6.71	5.72	6.56	5.95	5.85	6.49	6.67	5.70	6.21	6.71	5.70
9	5.76	6.11	6.81	5.91	5.91	6.86	6.78	6.44	6.32	6.86	5.76
11	6.58		6.81	6.73	5.37	6.81	6.58	6.04	6.42	6.81	5.37
Average..	6.40	5.55	6.27	6.38	4.88	6.48	6.60	5.99	6.09	6.74	4.66

Group Variations for Each Day.—The variations of the limits within which the lactose per cent is found show a fairly well marked tendency towards a narrowing. The maximum variation at the 11th day is only ±6 per cent from the mean, a much lower value than that observed for either protein or fat.

Group Variations from Day to Day.—These figures like the results of Camerer and Söldner, show from the quantitative standpoint an increase in lactose production for the period. Underlying these group variations there must be metabolic variations of an important nature.

Individual Variations from Day to Day.—These gradually become less and less and are finally confined within rather narrow limits.

Individual Variations from the Group Average for the Single Days.—The variation of lactose per cent production shows for the individual the same qualitative uniformity of production plane as is shown by both protein and fat.

Variations in the Constituents.

In an extended study of the daily variations in the milk constituents of the cow Hinchcliff (12) found that an increase or decrease in fat content was usually accompanied by an opposite change in the per cent of sugar or protein.

In order to study the variations in the constituents of human milk I have calculated the per cent variation for each constituent from day to day and tabulated them in Tables VII, VIII, and IX.

TABLE VII.

The Per Cent Variation in Protein Per Cent from Day to Day.

Days after parturition	Case.							
	1	2	3	4	5	6	7	8
3– 5	−18.6	−65.9	−60.4	−23.3	−54.4	−54.4	−13.7	−43.4
5– 7	− 8.0	− 7.1	−13.2	1.2	−17.1	−16.9	−14.1	27.1
7– 9	17.8	3.3	3.0	− 3.1	− 5.2	1.5	6.5	8.5
9–11	−16.1		− 6.5	− 9.0	−24.2	− 3.6	−19.7	− 4.5

TABLE VIII.

The Per Cent Variation in Fat Per Cent from Day to Day.

Days after parturition.	Case.							
	1	2	3	4	5	6	7	8
3– 5	8.2	− 1.4	−32.6	10.4	−71.0	6.8	−46.2	−10.6
5– 7	52.1	106.8	−35.1	40.6	−32.3	−1.2	102.6	84.6
7– 9	78.9	−28.3	25.9	−12.1	14.0	−7.9	6.5	− 3.2
9–11	−27.8		7.0	−26.7	8.1	1.3	23.0	−23.9

TABLE IX.

The Per Cent Variation in Lactose Per Cent from Day to Day.

Days after parturition.	Case.							
	1	2	3	4	5	6	7	8
3– 5	− 0.8	−34.6	41.7	4.6	114.7	13.9	12.3	16.3
5– 7	4.2	37.7	*	−12.6	17.9	− 0.5	−2.8	−10.0
7– 9	−14.2	6.8	3.8	− 0.7	1.0	5.7	1.6	12.9
9–11	14.2	†	*	13.9	− 9.1	− 0.7	− 3.0	− 6.2

* No change.

† No result.

Arbitrarily throwing out all variations less than 3 per cent, it is seen that for human milk it can be stated that *during the first 11 days after parturition with an increase or decrease in lactose production there is a general tendency towards the opposite change in fat or protein production.*

The most marked reciprocal relationship exists between the fat and lactose. Underhill and Baumann (19), in a series of comparative analyses of blood sugar and blood fat, have found

a similar relationship to exist in the blood of dogs poisoned by hydrazine. They find that, "blood fat shows an increase to a maximum which is coincident with the condition of hypoglycemia induced by hydrazine." That such an interdependency should exist in such widely divergent cases is worthy of note, and is significant of close relationship of fat and carbohydrate metabolism.

SUMMARY.

In summing up the variations observed in the chemical composition of human milk during the first 11 days after parturition, it is necessary to consider them both from the quantitative and from the qualitative points of view.

From the quantitative point of view fat and lactose increase in amount during the period studied, while there is a falling off in the production of protein.

If we accept as an index of variability the individual variation from the group average, and the individual variation from day to day, the three constituents fall into the following order of decreasing variability: *fat, protein, lactose.* This order is changed to *fat, lactose, protein,* when we consider the qualitative aspect in regard to the group changes in variation limits and the group uniformity of change in production direction.

The protein mechanism is apparently the best regulated and is less dependent upon the factors controlling the fat and lactose production than they are on each other.

For the single constituents there is a production plane uniform for the individual subject. This plane of production is fixed for the individual, independent of the plane of nutrition and dependent on the individuality.

An increase or decrease in the lactose production is generally accompanied by a change in the opposite direction in the per cent of fat and protein.[1]

[1] To the Superintendent of the Los Angeles County Hospital are due my acknowledgments of his kindness in allowing me to obtain the material for this piece of work. It was through the efforts of Dr. Lyle G. McNeile that the collection of the samples was efficiently carried out.

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THE RATE OF UREA EXCRETION.

III. THE EFFECT OF CHANGES IN BLOOD UREA CONCENTRATION ON THE RATE OF UREA EXCRETION.

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(Received for publication, January 22, 1917.)

On one side of the urea-secreting cells of the kidney there is blood and on the other side there is urine. The rate of urea excretion is the rate at which these cells transfer urea from blood to urine. Under these circumstances it seems probable that changes in the concentration of urea in the blood will have an effect on the rate of urea excretion.

If the concentration of urea in the blood were the only factor governing the rate of excretion, it would be possible to obtain a direct measure of its effect by determinations of the rate at different levels of blood concentration. But we have already shown[1] that the rate may vary when the blood concentration remains constant, and that there may be considerable differences in the rate, even when the concentration of urea in the urine as well as in the blood is constant. It is therefore certain that other factors besides the urea concentration of the fluids on either side of the kidney cells exercise a determining influence on the rate of excretion. And unless these other factors can be kept constant, any observed rate of excretion may be higher or lower than that which would have resulted had the blood concentration been the only operative factor.

We have not succeeded in framing experimental conditions under which we could be sure that this necessary constancy was attained. This of course was to be expected, since some factors which seem likely to influence the rate of work of the kidney

[1] Addis, T., and Watanabe, C. K., *J. Biol. Chem.*, 1916, xxiv, 203.

cells, as, for instance, the rate of flow of blood, could not be directly controlled in our experiments. But we have found that under certain conditions these unknown and uncontrolled factors tend to increase the rate of urea excretion above that usually found at the observed blood concentration, while under other conditions the rate tends to be decreased. Since these factors alter the rate in both directions, an average of a number of observations at different levels of blood concentration will give an approximation to the effect of blood concentration alone. The accuracy of this approximation will depend on the degree to which these observations represent an equal distribution between instances where the rate has been increased under the influence of other factors than blood concentration, and those in which it has been decreased.

The general method we have followed was to estimate the average blood urea concentration for intervals of 15 to 120 minutes during which the urine was collected. The rates of urea excretion were expressed as gm. of urea per hour. Points were plotted on a scale, so that the ordinates were equal to these rates, and the abscissæ to the corresponding measures of blood urea concentration. The curve drawn through these points will measure the effect of changes in blood urea concentration on the rate of urea excretion more or less accurately according as the above condition has been fulfilled.

The subjects were adults, at or below 35 years of age, who were free from any sign of kidney disease.

The higher levels of blood urea concentration were obtained by the administration of urea in doses of 20, 30, or 40 gm. of urea, the intermediate by observations taken before or after the concentration had reached its maximum following urea ingestion, while a considerable variety of lower concentrations was found in subjects who had taken no urea.

The conditions as regards food and water intake were very variable. In some experiments no food was taken for as long as 20 hours, in others considerable quantities of urea-forming food had been consumed. The water intake varied from no water for 12 hours or more before an experiment to quantities of 1,500 cc. before, and large quantities during the period of observation. No experiments in which drugs were given are included.

Collections of urine were made over periods which were made as short as was compatible with accuracy, and the average blood urea concentration for each of these periods was calculated. The error in the actual estimation of urea in the blood is small in comparison with the possible error which might arise in calculating an average concentration over a period of time from the concentration found during a few moments of that time. For the blood urea concentration is constantly changing, and it does not follow that the concentration found at the middle of a half hour period accurately represents the average concentration throughout the whole of that time. As a rule, however, changes in urea concentration rise or fall fairly evenly over periods of hours. The majority of our observations were made in consecutive series extending over 3 to 12 hours, and the blood urea was determined every hour or every half hour. In this way a curve of urea concentration was obtained and from this the average concentration for each period was calculated. This was particularly useful in those experiments in which urea was administered, for there the concentration rose and fell from a maximum. Where there was a smooth curve of this sort there was probably little error in the calculation of the average concentration for each period, except for that period in which the maximum was attained.

There is still another possible source of error in connection with the determination of the blood urea concentration. In experiments of the type we are concerned with, it seems to have been tacitly assumed that the concentration of urea found in blood removed from an arm vein is the same as the concentration which exists in the blood reaching the urea-secreting cells of the kidney. But since we know that urea is not secreted by the glomeruli, and have reason to conclude that a large part of the water of the urine is, it seems likely that the concentration of the blood which leaves the glomeruli is higher than that of the systemic blood in proportion to the amount of water abstracted from it in the glomeruli. In experiments in which the volume of urine is very large, it is conceivable that the difference thus produced might lead to appreciable error.

The estimation of urea in the urine was done in duplicate. As in the case of the blood urea, the greatest source of error is

probably not the technical one, which can be measured, but one arising from the conditions under which the experiments were conducted. Especially, we believe, in cases in which the urine volume was small, considerable error may sometimes have resulted from incomplete emptying of the bladder. The subjects, however, were asked to take special pains to make urination complete, and where the urine volumes were small, the periods over which the rate of excretion was determined were lengthened. In such cases more than one estimation of blood urea concentration was sometimes made for each period.

Part of the data has been given in tabular form in a previous paper,[1] though a considerable number of observations made since that time have been added.

All the observations made under these varying physiological conditions have been plotted in Fig. 1. The general trend reveals the existence of a relationship between the concentration of urea in the blood and the rate of urea excretion. The curve shows the theoretical quantitative effect of any given degree of change in blood concentration, other factors remaining constant. The degree of scattering indicates to what extent other factors than blood concentration may modify the rate of excretion.[2]

There are certain defects in the data which have to be conconsidered as probably detracting from the accuracy of the curve. Of these, the sources of error we have described above in the estimation of the rate of excretion and of the blood urea concentration cannot be considered as important in relation to the extent of the scattering seen in the graph. Appreciable errors were certainly only occasional, whereas the scattering is general. The most important and probable cause of a deflection of the curve from its true course would be a want of balance between instances of acceleration and of depression of the rate of excretion due to other causes than changes in blood concentration. The drinking of large quantities of water tends to accelerate the rate of excretion, and abstention from water to decrease it, apart from any alteration in blood concentration. There is a fairly even distribution of observations made under

[2] The curves were drawn for us by Mr. Otis, of Stanford University, by means of a method he has described which is called the "method of rank correspondence." Otis, A. S., *School and Society*, 1916, iv, 716, 750.

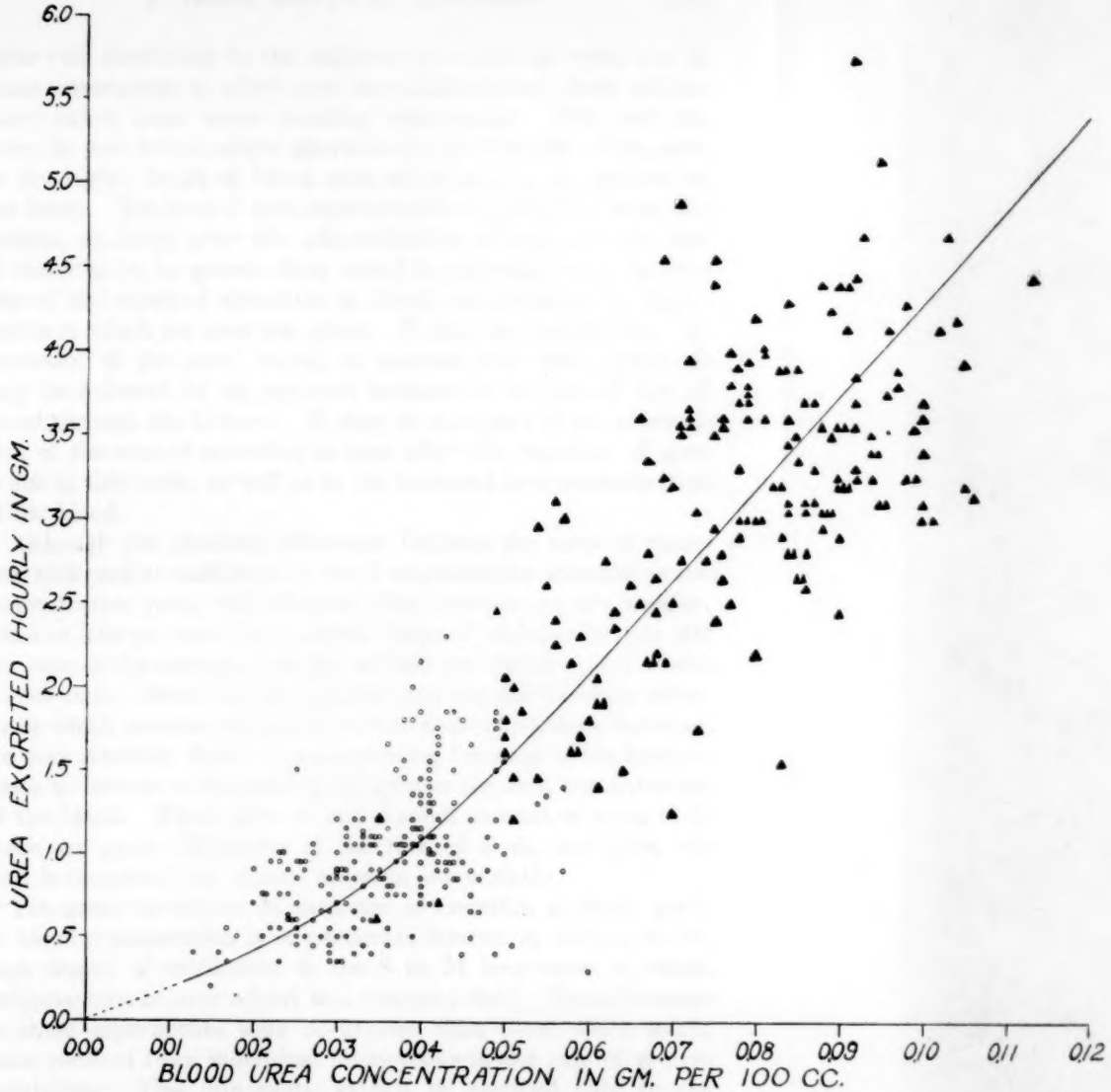

FIG. 1. The effect of changes in blood urea concentration on the rate of urea excretion. Observations on a group of individuals. The circles indicate experiments in which no urea was given, and the triangles those in which urea was administered.

these two conditions in the subjects who took no urea, but in those experiments in which urea was administered, there are too many taken from water drinking experiments. This may account in part for the slight alteration in the direction of the curve at the higher levels of blood urea concentration as opposed to the lower. Yet even if such experiments are excluded, there is a definite tendency after the administration of urea for the rate of excretion to be greater than would be expected from observation of the result of alteration in blood concentration in experiments in which no urea was given. It has been noted from observation of the renal vessels in animals that urea injections may be followed by an apparent increase in the rate of flow of blood through the kidneys. It may be that part of the acceleration of the rate of excretion in man after the ingestion of urea is due to this cause, as well as to the increased urea concentration of the blood.

Although the absolute differences between the rates of excretion observed at each level of blood concentration increase as the concentration rises, yet relatively they become steadily smaller. Thus at 0.03 per cent the probable range of variation is over 200 per cent of the average, whereas at 0.09 per cent it is only about 75 per cent. Since it is the relative and not the absolute differences which measure the degree of variability in kidney function, we may conclude that the urea-excreting function of the kidneys tends to become more uniform the greater the urea concentration of the blood. When there is only a small amount of work to do there are great differences in the rate of work, but when the load is increased, the output becomes less variable.

The great variability in the rates of excretion at every grade of blood concentration is of particular interest in relation to the high degree of uniformity in the 8 to 24 hour rates at which subjects excrete urea added to a constant diet.[3] The differences in these experiments were no greater than those which might have resulted from individual fluctuations in the rate of protein catabolism. This uniformity cannot be ascribed only to the equality of conditions as regards food and water intake, for we have recorded instances[1] in these subjects where the rates of

[3] Addis and Watanabe, *J. Biol. Chem.*, 1916, xxvii, 249. There was no egg given with the noonday meal, as is incorrectly stated in this paper.

excretion during short periods of time showed a variation not markedly lower than that shown in Fig. 1, even when the blood concentration was constant. We believe that the uniformity arose mainly from the fact that the rate was observed over long periods of time (8 to 24 hours), while in the experiments given in this paper the time of observation was relatively short (15 to 120 minutes). Though kidneys which are called on to perform equal amounts of work may vary their rate of work widely from hour to hour, their total performance for the day is relatively uniform. If this is the true explanation, we may conclude that those unknown factors which cause the kidney to alter its rate of work have only a temporary and evanescent effect, and that a depression of kidney activity during 1 hour of the day is counterbalanced by a later acceleration, so that the total output remains the same.

The fact that in twenty-nine young adults a high grade of uniformity was found in the 8 to 24 hour rate of excretion of administered urea[3] indicates that there were no fixed individual peculiarities in the kidneys of that group. The subjects of the present observations include all of the above group, and the additional subjects were similar in their age and freedom from evidence of kidney disease. It seems therefore improbable that the scattering shown in Fig. 1 can be due to permanent individual differences in kidney structure. But it is possible to give direct evidence in regard to this point from our data. A considerable number of observations (110) were made on one subject under all the various experimental alterations in food and water intake. Fig. 2 shows the curve and the degree of scattering in his case. Since the kidneys of this individual show as great a variability as is manifested by the kidneys of all the other subjects, we may conclude that permanent anatomical differences are not responsible for the variations in the rate of urea excretion independent of changes in blood concentration during short time intervals. The causes of these variations must be sought for among temporary, irregular, and counterbalancing alterations in the environment of the kidney.

In the next paper evidence is presented showing that alterations in the urea concentration of the urine play no appreciable part in the production of these variations.

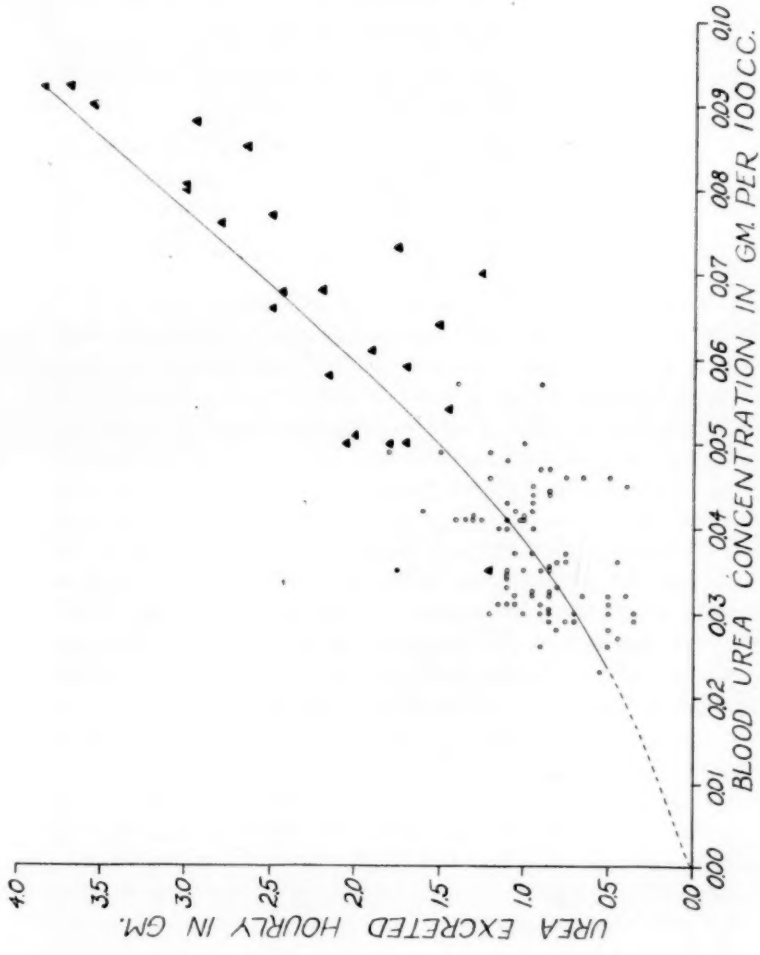

FIG. 2. The effect of changes in blood urea concentration on the rate of urea excretion. Observations on a single individual.

CONCLUSIONS.

1. A curve has been constructed which indicates the effect of changes in blood urea concentration on the rate of urea excretion in man.

2. The wide scattering of the observations from which the curve was constructed shows that other factors than blood urea have a pronounced effect on the rate of urea excretion.

3. There is a relative decrease in the degree of scattering, as the blood urea concentration increases. This is interpreted as indicating that the greater the stimulus to increased work in excreting urea, the less subject the kidney becomes to influences tending towards variability in its rate of work.

4. The variability in the rate of urea excretion at every level of blood urea concentration during the short periods of time chosen in these experiments is in marked contrast to the uniformity in the rate of excretion of administered urea over periods of 8 to 24 hours. This is taken as indicating that unknown factors lead to short-lived variations in the rate of excretion, and that these variations tend to counterbalance one another over longer periods.

5. The degree of scattering in one individual is as great as the scattering in the whole group. This makes untenable the hypothesis that permanent individual peculiarities, such as might arise from anatomical differences in kidney structure, are responsible for the variability in the rate of urea excretion revealed by the scattering shown in Fig. 1 for the group.

THE RATE OF UREA EXCRETION.

IV. THE EFFECT OF CHANGES IN THE VOLUME OF URINE ON THE RATE OF UREA EXCRETION.

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(Received for publication, January 22, 1917.)

Since the concentration of urea in the blood influences the rate at which urea passes through the urea-secreting cells of the kidney, it seems likely that the concentration of urea in the urine in contact with the internal surfaces of these cells should also have an effect. A high concentration would tend to delay, and a low concentration to facilitate the discharge of urea from the cells, at least if the mechanism of this discharge depended on a process of diffusion. It is, of course, obvious that we cannot determine the actual urea concentration of the urine which lies next to the urea-secreting cells. The anatomical evidence given by Leschke,[1] which has been confirmed by Oliver,[2] demonstrates that urea is secreted by the cells of the proximal convoluted tubules. Since there is every reason to believe that a great part of the water of the urine passes through the glomeruli, we should expect that a series of different concentrations, increasing in proportion to the distance from the glomerulus, will be present along the reaches of each proximal tubule. And when, further, the possible occurrence of secretion and absorption of water at lower levels of the tubular system is remembered, it becomes apparent that we cannot assume any close or constant relationship between the urea concentration in urine collected from the bladder, and the urine as it lies in the proximal tubules. We have grounds, however, for believing that a rough general parallelism exists, and that when the urea concentration of collected urine is found to be greatly

[1] Leschke, E., *Z. klin. Med.*, 1914, lxxxi, 14.

[2] Oliver, J., *J. Exp. Med.*, 1916, xxiii, 301.

decreased as the result of an increase in volume produced by water drinking, there will also be a decrease in the average concentration in the proximal tubules.

In observations on the volume of urine of subjects under constant dietary conditions,[3] we found that the urine volume was increased when the rate of urea excretion was approximately doubled by the administration of urea. On the other hand, the rate of urea excretion was not increased when the urine volume was doubled by the administration of water. This raises the question as to whether there may not be a general relationship between rate of urea excretion and volume of urine of such a nature that the urea excretion influences the volume, in contradistinction to the idea of the volume influencing the urea, which is implied in the above mentioned theoretical considerations as to the possible effect of the urea concentration of the urine.

With the data at our disposal, we can determine whether there is evidence of any general relationship between the volume of urine and the rate of urea excretion, such as would lend support to either of these hypotheses. This might be accomplished by determining the net correlation between these two factors by means of a formula for partial correlation. But it was pointed out to us that the distribution in our material of the three variables (rate of urea excretion, volume of urine, and concentration of urea in the blood) was so far from normal as to render any coefficient of correlation somewhat misleading. It was therefore considered better to eliminate the factor of blood urea concentration by plotting the rates of urea excretion as plus or minus differences between the observed hourly rates and those which the curve shown in Fig. 1 of the preceding paper indicated as normal for the blood concentration found in each instance.

These differences are represented in Fig. 1 by the ordinate, while the abscissa measures the volume of urine per hour. It is apparent that there is no general relationship between the volume of urine and the rate of urea excretion, since there is no indication that the minus values occur predominantly with low volumes of urine, nor any clear tendency towards a gradual increase over the normal value to successively greater plus values

[3] Addis, T., and Watanabe, C. K., *J. Biol. Chem.*, 1916, xxvii, 267.

FIG. 1. The absence of any direct relationship between changes in the volume of urine and the rate of urea excretion. Observations on a group of individuals. The circles indicate experiments in which no urea was given, and the triangles those in which urea was administered.

as the urine volume increases. This absence of relationship between volume and rate of excretion means that there is also no relation between the concentration of urea in the urine and the rate of excretion, because this concentration is the amount of urea in 1 hour's urine (the rate of excretion) divided by the hourly volume of urine.

In a recent paper[3] we said we had evidence that under certain conditions the volume of urine was an important factor in determining the rate of urea excretion. The conditions we had in mind were those in which the volume of urine was unusually low because of abstention from water and was then suddenly increased by copious water drinking. We found that concomitantly with the rise in urine volume there was an acceleration of the rate of urea excretion which was not accounted for by any increase in blood urea concentration. But a more complete analysis of the figures showed such discrepancies between the degrees of increase in rate of urea excretion and volume that there cannot be said to be any indication of a causal relationship between them. In Table I we give the details of an experiment carried out under these conditions.

During the first period of this experiment, the hourly volumes of urine were small because the subject had taken no water or food for 12 hours. At the commencement of the second period 20 gm. of urea were taken in a concentration at about the maximum concentration the normal kidney is capable of secreting. This led to a call for an increase in the rate of urea excretion under conditions in which there would tend to be a relative insufficiency of water available for carrying out the added urea. During the third period the body was flooded with water by the ingestion of 1,500 cc. and the drinking thereafter every quarter of an hour of amounts of water equivalent to the volumes of urine excreted.

The outstanding feature of the experiment is the increase in the rate of urea excretion which accompanied the rise in urine volume induced by water drinking. The rates which during the first and second periods were all lower than the normal as given by the curve become higher than normal during the third period. There can be no question from the results of this and other experiments that abstention from food and water tends to lower, and the drinking of large quantities of water to increase the rate,

independently of the blood urea concentration. On the other hand, the want of parallelism between the volumes and the differences between the actual and normal rates is so complete that we cannot say that the rates increase because of the rise in urine volumes, or that the volumes increase because of the rise in the rate of urea excretion. The greatest increase over the normal rate occurs when the volume of urine is still not unusually large while when the diuresis is at its maximum the rate is less

TABLE I.

Period.	Actual urea excretion per hr.	Blood urea concentration per 100 cc.	Normal urea excretion for the blood concentration found.	Difference between the actual and the normal urea excretions.	Volume of urine per hr	Times between which urine was collected.
	gm.	*gm.*	*gm.*	*gm.*	*cc.*	
1. Nothing taken. No food or water during the previous 12 hours.	0.47	0.036	0.92	−0.45	29	9.42–10.45 a.m.
	0.38	0.029	0.65	−0.27	25	10.45 a.m.–12.45 p.m.
	0.48	0.030	0.70	−0.22	26	12.45–2.00 p.m.
2. At 2 p.m. 20 gm. of urea were taken in 5.6 per cent solution.	1.50	0.064	2.31	−0.81	68	2.00–3.00 p.m.
	1.25	0.070	2.65	−1.40	41	3.00–4.00 "
3. At 4 p.m. 1,500 cc. of water were taken and thereafter a quantity equal to the volumes of urine excreted.	2.37	0.067	2.48	−0.11	70	4.00–4.30 p.m.
	3.67	0.066	2.42	+1.25	120	4.30–4.45 "
	2.86	0.066	2.42	+0.44	176	4.45–5.00 "
	2.64	0.066	2.42	+0.22	352	5.00–5.15 "
	2.40	0.066	2.42	−0.02	440	5.15–5.30 "

than normal. Similar discrepancies are noted when the changes in the urea concentration of the urine are compared with the rate. Thus both the highest (+) and the lowest (−) rates occur in periods during which the urine concentration is 3 per cent.

We no longer believe, therefore, that we are justified in tracing a direct relationship between the volume of urine and rate of urea excretion. All that we can say is that conditions accompanied by low urine volumes are also frequently associated with

subnormal rates of urea excretion and that when a large quantity of water is drunk the rate of excretion may become greater than normal as the urine volume increases. But these two phenomena appear to be independent of one another. Both may be the result of some common cause, such as an alteration in the blood circulation through the kidneys or a change in the activity of the kidney cells. If changes in the free water content of the blood have an effect on urine volume comparable to that which alterations in its urea concentration have on the rate of urea excretion, it might be possible to demonstrate a parallelism between variations from normal volumes and normal rates under the influence of some such common cause.

CONCLUSIONS.

1. Changes in the volume of urine or in the urea concentration of the urine have no appreciable effect on the rate of urea excretion.

2. When no food or water have been taken for some time, the drinking of large quantities of water is followed by an increase in urine volume and by a synchronous acceleration of the rate of urea excretion which cannot be accounted for on the basis of changes in blood urea concentration. But there is no evidence that this increased rate is a result of the increased volume of urine for the degree of increase above the normal in the rate is quantitatively independent of the degree of increase in volume.

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